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SEMIANNUAL REPORT

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National Aeronautics and Space Administration
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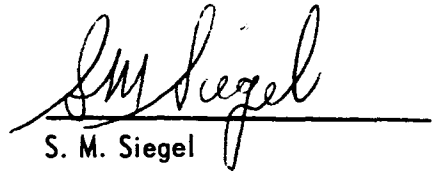
UNION CARBIDE RESEARCH INSTITUTE
Tarrytown, New York

Laboratory of Union Carbide Corporation

Submitted by:



S. M. Siegel
Principal Investigator

National Aeronautics and Space Administration
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I. SURVIVAL OF TERRESTRIAL ORGANISMS UNDER ULTRAVIOLET FLUXES OF 400,000,000 Erg-cm^{-2} OR MORE

Electromagnetic energies in the near ultraviolet (ca 2000-3000 Å, 50-90 kcal/Einstein) are well known for their biological and chemical effects. Radiation in the region of 3000 Å reaches the earth's surface and produces in man and other animals erythema, tanning, and in extreme cases contributes to death from exposure. Radiation in the mercury resonance region, 2537 Å, has long been used to produce general lethal effects as well as mutations.

Notwithstanding the impressive effects of ultraviolet radiation, it does not penetrate readily through solids or aqueous solutions containing halides, heavy metal salts, or organic matter. Furthermore it has been held that superficial pigmentation in organisms serves as an effective ultraviolet screen. Nevertheless, Evans has recently completed a study of martian ultraviolet reflectivity and concludes that since 90% of direct solar radiation between 2000 and 3000 Å, reaches the surface of Mars, "...a lethal exposure to the radiation would be accumulated in 1 or 2 days for almost all types of bacteria, spores, fungi, viruses, protozoans, and so forth found on earth."

This unfortunate near generalization has reached the popular scientific level in the form of the statement, "Strong ultraviolet light on Mars would prevent any earth-type life from existing on the planet's surface..." which is even more of a generalization.

The present communication is addressed neither to the subject of general simulation of martian environments nor to the nature of martian lifeforms, but to the correction of an overestimate of ultraviolet radiation as an eliminative environmental factor.

The organisms used in tests of ultraviolet (UV) effects included relatively complex animals and plants as well as microscopic forms.

Animal species included: Bursaria sp. (Ciliata), Cerastium sp. (Dinoflagellate), Monostyla sp. and Philodina sp. (Rotifera), Dugesia and other Planarians, Chlorohydra sp. (Coelenterate), Cephalobus sp. and Anguillula sp. (Nematodes), Iulius sp. (Millipede), a mixed isopod culture, a tarantula of undetermined species, Tenebrio molitor (Insecta), and Pseudemys scripta-elegans (Reptilia).

Vascular plants tested were: Begonia hybrida, Coleus blumei, Aloë variegata, Faucheria tigrina, Hedera baltica, Pleiospilos nelii, Portulacaria sp., Sedum sp., Sempervivum tectorum, Stapelia sp. and spores of Pteris longifolia. Except for the fern spores, mature plants were used in survival tests.

Other forms included Cladonia rangiferina, Rhizopus nigricans, Physarum polycephalum, Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Staphylococcus lactis, and several pseudomonads. Bacteria were irradiated after 24 hr growth at 30°C on standard nutrient agar plates. Following UV treatment, fresh plates were streaked from control and treated surfaces and incubated 6 days in darkness at 30°C. Spores of fungi and sclerotia were irradiated dry, then placed on growth media at 25°C in laboratory light of ca 100 ft-c intensity.

The principle UV source employed was the "spectroline" 11SC-1 quartz pencil or a B4T4 low pressure mercury tube, both emitting over 85% at λ 2537 Å. In a few experiments, a Westinghouse 15-watt fluorescent tube emitting from 2750-3800 Å, maximally at 2900-3100 Å was used. Most experiments were carried out in air, but on a few we used N_2. Ozone build up under 2537 Å was prevented

by an air stream purge at ca 10 liters per minute. In experiments with turtles (Pseudemys) in 100% O_2, an alkaline arsenite screen was used to eliminate O_3.

Inasmuch as we challenged the assertion that the accumulated dosage of UV on Mars would be lethal in 1 or 2 days, experimental exposures were reckoned as multiples of the calculated daily flux on Mars for an assumed 12 hr per 24 hr diurnal cycle. This figure was obtained by correcting the flux density at the top of the earth's atmosphere in the interval λ 2537 Å (assigning the entire energy to λ 2537 Å) for the greater mean distance of Mars from the sun (1.52 astronomical units), and correcting for atmospheric attenuation using Evans figure of 90% transmission. The results of calculation gave 4.32×10^8 erg-cm^{-2} as the 12-hr daily dose. This corresponds, in turn to a dose rate of about 10^5 erg-cm^{-2} sec^{-1}, whereas we used continuous irradiation in general at a dose rate of over 4×10^5 erg-cm^{-2} sec^{-1}. Our treatments were, therefore, more severe than a comparison of total dosage per unit area requires.

A. Animal Tests

Of 12 assorted species tested, 5 failed to survive a single equivalent martian day (EMD). These included a protozoan, a nematode, a coelenterate, a rotifer and assorted planarians (Table I). These data cannot be considered representative for the groups involved, only for the species used. This point is shown clearly by comparing the rotifer Monostyla which failed, with Philodina, which had motile survivors after ca 8 EMD. Further, Anguillula was only slightly impaired by ca 5 EMD exposure, whereas the other nematode, Cephalobus succumbed to less than one EMD. Outstanding resistance was shown in the isopods (under a 15 mm pond water layer), a tarantula and a beetle. All survived doses of at least 11 EMD. The turtle Pseudemys was tested for only 0.7 EMD, but under especially rigorous conditions - in 760 mm O_2. Individual turtles

Table I

Survival of Various Animals

Exposed to λ 2537 Å Radiation[a]

Organism	Dose Erg-cm^{-2} x 10^8	Equivalent No. Martian Days[d]	Response No. Survivors/No. Total
Angiullula	76	17.7	2250/3000
Bursaria	< 4.3	< 1	0/> 1000
Cephalobus	< 4.3	< 1	0/2500
Cerastium	6.5	1.5	80/1000
Chlorhydra	< 4.3	< 1	0/25
Isopods	6.5	1.5	38/40
	39	9.1	30/40
	140	32.6	2/40
Monostyla	< 4.3	< 1	0/600
Philodina	6.5	1.5	39/500
	39	9.1	8/500
	120	27.9	2/500
Planaria	< 4.3	< 1	0/75
Pseudemys[b]	ca 10	ca 2.3	10/10 (in 100% O_2)
Tarantula	140	32.6	2/2
Tenebrio (Beetle)	140	32.6	1/1
	6.5[c]	1.5	2/7

[a] Rate 4.2 x 10^4 erg-cm^{-2} sec^{-1}

[b] In O_2 at 760 mm Hg

[c] At λ 3000 Å, rate 9 x 10^2 erg-cm^{-2} sec^{-1}

[d] 1 EMD = 4.3 x 10^8 erg-cm^{-2}

ventured quite close to the radiation source, and responded by extending their heads, eyes open, toward the UV tube. No sign of visual impairment was evident immediately after irradiation or during several subsequent months under normal conditions. The spiders also exhibited a strong positive phototaxy toward the UV tube, actually grasping it with one or more pairs of legs for as much as an hour at a time.

B. Plant Responses

The general criteria for UV damage to mature vascular plants are browning of pigmented tissues, general liquescent appearance in patches, especially in succulents, shrivelling of leaves, acute loss of tugor, abscission and collapse. Using a standard dose of UV, ca 8 EMD, only minor signs of injury at most were evident in some of the plants in an N_2 atmosphere (Table II). Exceptions were Coleus and Begonia which were acutely damaged. Faucheria showed a slight drop in turgidity and Portulacaria abscission in older leaves only. Three species showed color changes which differed from those indicative of damage. They produced pink or brown pigments overlaying normal green. These appear to be phenolic in character and are now under study.

C. Survival of Micro-organisms

Since most studies in the area of ultraviolet toleration have been carried out with micro-organisms, only a few species were included for comparative purposes to demonstrate this group's stress capabilities. Of the organisms tested, those growing after periods of more than 7 EMD were: B. subtilis, E. coli, Ps. aeruginosa, and Ps. spp ATC 11299a.

Spores and sclerotia of fungi exposed to more than 2 EMD of ultraviolet showed normal germination and subsequent vegetative growth.

Table II

Survival of Vascular Plants Exposed to 10^{10} erg-cm^{-2} of λ 2537 Å Radiation in 760 mm N_2[a]

Species	Response after 72 hr Continuous UV
Aloë	Leaves green and turgid.
Begonia	Leaves orange brown, blistery.
Coleus	Leaves limp, discolored.
Faucheria	Leaves somewhat flaccid, but green with pink spots.
Hedera	Leaves intact, dark green.
Pleiospilos	Body turgid, UV-side pink.
Portulacaria	Younger leaves intact, but oldest leaves fallen.
Sedum	Leaves green, turgid.
Sempervivum	Leaves green, turgid.
Stapelia	Body upright, firm, UV-side brown.

[a]ca 23 equivalent martian days

D. The Oxygen Effect

The relationship between UV radiation and oxygen has not received the attention given to the interactions between O_2 and X- (or γ-) radiation. The response shown by turtles would in fact weaken any general case for such an interaction. Nevertheless, among the few systems studied under UV thus far, it has been possible to demonstrate some marked O_2 dependencies (Table III). The UV-sensitive nematode Cephalobus is less sensitive after gassing with N_2 at 760 mm pressure for 30 min. prior to irradiation. The LD_{50} for Cephalobus in air is, roughly $0.4\text{-}0.5 \times 10^6$ erg-cm^{-2}, whereas under N_2, the figure is approximately doubled to about 1×10^6 erg-cm^{-2}. The lichen Cladonia shows a marked metabolic response to UV, namely a 3-fold elevation in Q_{CO_2} during exposure in air, and a return to the normal dark value immediately upon removal of the UV source. In contrast, under N_2, there is essentially no change in Q_{CO_2} whether the lichen is exposed to UV or not. Among vascular plants, one of the most striking distinctions between exposure to UV in air or N_2 is in defoliation. Without O_2, defoliation is limited to older leaves at most, but in air, the same UV dosage induces the abscission of virtually every leaf, save perhaps for the youngest. Thus the defoliating activity of UV, like that of ethylene, is O_2-dependent.

Table III

Evidence for an Oxygen Effect in Ultraviolet Damage*

a. Cephalobus

(λ 3000 Å at 9×10^2 erg-cm^{-2} sec^{-1})

Dose (erg-cm^{-2} x 10^6)	Survival (%) Air	N_2
0.7	20	100
1.4	0	10
2.1	0	0

b. Cladonia

(λ 2537 Å, 3.6×10^9 erg-cm^{-2})

Atmosphere	Ultraviolet	CO_2 Production mm^3 gm^{-1} hr^{-1}
Air	off 24 hr	64 ± 4
	on 24 hr	190 ± 18
	off 24 hr	57 ± 4
N_2	off 24 hr	57 ± 6
	on 24 hr	69 ± 3
	off 24 hr	56 ± 5

c. Vascular Plants

(λ 2537 Å, 10^{10} erg-cm^{-2})

Species	Defoliation Air	N_2
Hedera	Complete	None
Portulacaria	Complete	Older leaves only
Sedum	Complete	None

* All atmospheres at 760 mm pressure.

The existence of an oxygen effect adds an important parameter to any consideration of UV on an environmental basis, because some organisms which succumb in air will not do so when living as anaerobes. The conditions of existence in many locales on earth, Mars, or elsewhere involve still other factors such as temperature and water which will further modify the response to UV.

Oxygen is of particular interest in relation to other stress factors, because, in addition to the present demonstration, it has been shown to modify the levels of heat, cold, and ionizing radiation which organisms can withstand.

In addition to the conclusions bearing upon UV-flux and surface life on Mars which we have questioned, it has also been maintained that prior to the release of large quantities of O_2 by photosynthesis, the lack of an O_2-derived ozone layer would have (again) permitted a lethal level of UV to reach the earth's surface, restricting life to zones many meters below the surface of the late pre-Cambrian oceans. The authors feel that the paleobiological conclusion, while better founded and developed than the exo-biological, nevertheless must be tested on biological grounds. Thus, the absence of O_2, hence ozone, while permitting intense UV to reach the surfaces of the land and sea, may have in itself limited the effectiveness of the UV as an acute stress factor.

The data which have been presented show that limited doses of ultraviolet radiation are not necessarily lethal toward organisms representing a wide range of organizational levels and evolutionary affinities. Even if a majority of terrestrial life forms were to be killed by martian UV levels, generalizations about the indigenous life-forms of that planet would be insupportable.

II. GERMINATION OF *ALLIUM* IN AMMONIA-RICH ENVIRONMENTS

The oxides and hydrides of nitrogen are physiologically active and usually toxic to most forms of plant and animal life. The mammalian toxicity of NH_3, for example, is comparable with that of carbon monoxide. In recent work in this laboratory a variety of organisms have revealed unexpectedly good capabilities for growth under NO, NH_3 and other nominally toxic gases (Table IV).

When seeds were incubated under NH_3 (50% by volume in air), it was found that germination was totally and irreversibly inhibited in various members of Graminae (wheat, rice, rye), Compositae (lettuce, sunflower), Leguminosae (bean, pea), Cruciferae (turnip, cabbage, radish), Labiatae (mint, sage, coleus) and representatives of other Angiosperm families.

In spite of this formidable record of phyto-toxicity, NH_3 failed to inhibit completely the germination of *Allium* seed populations. The resistant fraction, under 15 per cent of these populations, obviously constitutes a physiologically distinctive experimental group.

Seeds representing 25 cultivars of onion (*Allium Cepa*) and four of leek (*A. Porrum*), all from the 1965 commercial crop, and wild onion *A. cernuum*, collected in Virginia in 1965, were used experimentally. Seeds were incubated on wet filter paper in petri dishes at 22°C under ca 50 ft-c daylight fluorescent illumination. Experiments with ammonia atmospheres were carried out in the petri dishes in 4 ℓ jars that had been evacuated three times and refilled with ammonia. When mixtures containing ammonia were used, the second gas was introduced first, and adjusted to the desired pressure. Ammonia was then added to give a steady gauge reading at a total pressure of 760 mm. This required repeated addition of anhydrous NH_3 until the aqueous phase was saturated. When a composition including NH_3 is described,

Table IV Examples of Organisms Grown in Atmospheres Containing Oxides or Hydrides of Nitrogen

Atmospheres	Pressure (mm. Hg)	Organisms Grown
100% N_2O	760	rye*
16% NO + 84% N_2	75	sorghum, rye, rice
100% NO	760	soil bacteria, yeasts
16% NO_2 + 84% N_2	75	sorghum, rye, rice*
5% NH_3 + 45% H_2 + 50% CH_4	760	Pseudomonas, Helminthosporium
50-95% NH_3 + CH_4	760	Torula, Saccharomyces, Penicillium, Clostridium
27% NH_3 + 27% CH_4 + 10% O_2 + 36% N_2	760	Kakebekia,** blue-green algae, myxobacteria

* Stimulated relative to 100% N_2 at the same total pressure.

** Found only in a specific soil sample, an obligate ammonophile.

reference is made only to the gas phase, neglecting the NH_3 required to saturate the aqueous phase.

An incubation period of 4 days was selected because all of the seeds capable of germinating in the presence of ammonia were found to do so within that time. In contrast, complete germination in water required approximately 7 days' incubation, but exceeded 50% for most cultivars by the end of day 4. A seed was considered germinated when a firm, white radicle had emerged at least 2.5 mm. After 4 days, all seedlings had 2.5-4 mm radicles. Seeds which failed to germinate after 4 days in any experimental level of NH_3 did not germinate when washed and returned to ordinary aqueous media in air for 14 days or more.

At a minimum, germination percentages were based upon duplicate determinations each with 50-75 seeds. In the survey of varietal differences (cf Table V), figures averaging one per cent or less were based upon replicates totalling nearly one thousand seeds.

A. Survey of Cultivars

Comparative germination data were obtained for the entire array of species and varieties in a 50% NH_3 atmosphere under aerobic and anaerobic conditions (Table V). In $NH_3 + N_2$, twenty onion and four leek cultivars germinated to some extent. Upon replacement of N_2 with air, several forms that failed to germinate anaerobically yielded seedlings, but several anaerobic varieties failed completely when incubated with aerobic ammonia. In general, those populations yielding substantially more than one per cent germination – 'Sweet Spanish', 'White Sweet Spanish', and 'Evergreen Long White Branching' onion, plus all of the leeks - showed little distinction between aerobic and anaerobic conditions.

Table V Germination of *Allium* Cultivars in Ammonia-Rich Atmospheres

Cultivar	Percent Germination* After 4 Days in 50% NH_3 + 50% N_2	50% Air
Onion (*A. Cepa*)		
Yellow Bermuda	0	0
Sweet Spanish Hybrid	0	1.3
Excel Bermuda	0	0
Burpee Yellow Globe Hybrid	0.7	0.8
Crystal White Wax	0.5	0
Southport White Globe	1.1	1.2
Red Wetherfield	0	0.6
Ebenezer or Japanese	0.6	0.5
Sweet Spanish	6.1	3.4
Australian Brown	0.9	1.3
Early Grano	6.1	3.8
White Sweet Spanish	5.1	5.0
White Portugal	1.4	3.1
Yellow Globe Danvers	1.5	1.3
Southport Red Globe	0.5	2.2
Southport Yellow Globe	1.6	0.9
Evergreen Long White Bunching	7.6	8.4
Early Yellow Globe	1.4	0
Autumn Spice F_1 Hybrid	1.4	0
He Shi Ko	3.3	3.0
Magnifico Hybrid	1.6	1.1
Downing Yellow Globe	1.1	0
White Lisbon	1.2	3.7
Bermuda Crystal White	0	0.4
White Portugal Bunching	2.0	1.6

* Each number is based on at least 800 seeds germinated 4 days at 22°C.

Table V (Cont'd)

Cultivar	Percent Germination* After 4 Days in 50% NH_3 + 50% N_2	50% Air
Onion (A. cernuum)		
wild onions	0	0
Leek (A. Porrum)		
Elephant	5.8	5.8
Large Musselburg	6.5	5.0
Broad London or American Flag	7.3	9.3
Large Flag	11.4	7.6

* Each number is based on at least 800 seeds germinated 4 days at 22°C.

Of the thirty Allium species and cultivars tabulated, only ten have been tested for germination under nitrogen anoxia: 'Yellow Bermuda', 'Sweet Spanish Hybrid', 'Excel Bermuda' and wild onion were obligate aerobes. 'Southport Yellow Globe', 'Evergreen Long White Bunching', 'White Portugal Bunching', 'Crystal White Wax', 'Yellow Globe Danvers' and 'American Flag' leek were facultative. Of the four obligate aerobes, three failed to germinate under 50% NH_3 + 50% N_2, but one, 'Sweet Spanish Hybrid'; germinated well. Of six facultative aerobes (2-4% germination in 100% N_2), all germinated ranging from 0.5 to 7.3% in the NH_3-N_2 mixture.

This response of Allium obviously involved more than imbibitional swelling, and would be of interest whether it was based wholly upon cell expansion, upon cell division, or both. Smears fixed and stained in aceto-orcein showed that 4-day old 'American Flag' leek roots consist of about 13% dividing cells (based on ca 230 cells examined) when grown in air, and ca 5% dividing cells (based on 430) when germinated in 100% ammonia. Comparable figures of ca 14% in air and 4% in ammonia were given by 'Evergreen' onion roots. The chromosomes of root cells from seeds germinated under NH_3 were, however, abnormal, having the appearance of highly attenuated, elongated spirals. Metaphase was especially poorly defined and even interphase nuclei were atypical, displaying a red-orange rather than typical magenta color.

B. Atmospheric Compositions

Two onions and two leeks were chosen for a more systematic study of variations in ammonia compositions. The presence of 10-25% NH_3 increased germination moderately to markedly above the level found under pure nitrogen anoxia (Table VI). This response is sustained at higher NH_3 levels

Table VI Effect of Variations in NH_3 Upon Anaerobic Germination of *Allium*.

Atmosphere (Vol. %)		*Allium* Cultivar (% Germination)			
		A. Cepa		*A. Porrum*	
NH_3	N_2	Evergreen	White Portugal	Large Flag	American Flag
0	100	4.0	0	0	0
10	90	9.0	1.0	10.0	11.1
25	75	5.4	1.0	8.0	5.0
50	50	8.0	0	8.0	9.0
100	0	8.0	1.0	4.0	5.0

although both leek cultivars showed a tendency toward decline above 50% NH_3. However, when ammonia was held constant at 50 vol % and the O_2 level varied from none (50% N_2) to 50% (no N_2), no such trends were evident (Table VII).

C. Other Bases

In an effort to sort out the factors in NH_3 toxicity and tolerance, comparisons were made with four other classes of substance; (1) related nitrogen bases, (2) strongly alkaline non-nitrogenous base (3) a non-alkaline ammonium salt, and (4) near neutral Na salt (Table VIII). Considering the unexpected ability of Allium to germinate in ammoniacal media which initiated this work, it was no surprise to see even better germination in hydrazine, a slightly weaker base, or methylamine, a somewhat stronger base than NH_3.

Germination was markedly greater in 0.01 M NaOH than in ammonia or hydrazine. 'Evergreen onions', in fact, gave somewhat higher germination in 0.1 M NaOH at pH 13 than in NH_3 at pH 12.2. 'American Flag' leek, however, failed completely at pH 13. In all other respects the behavior of onion and leek in basic media far exceeded expectations. Slightly acidic ammonium acetate and molar sodium chloride prevented germination completely.

One striking difference between 0.01 M NaOH, pH 12, and the other basic media was the appearance in the alkali solution of green shoots over 1 cm in height after about 7 days.

D. Penetration of Ammonia

No quantitative studies of NH_3 uptake have been attempted, but a few qualitative tests showing ammonia entry have been carried out successfully.

Table VII Effect of Variations in O_2 Upon Germination of *Allium*

Atmosphere Vol. %			*Allium* Cultivar (% Germination)			
			A. Cepa		*A. Porrum*	
O_2	NH_3	N_2	Evergreen	White Portugal	Large Flag	American Flag
0	50	50	7.6	1.4	11.4	7.2
0.5	50	45.5	6.0	2.0	10.0	9.0
5	50	45	6.0	0	6.0	8.0
10	50	40	8.4	3.1	7.6	9.3
50	50	0	8.0	1.0	10.0	6.0

Table VIII Germination of Allium Seed in Nitrogen Bases and Other Alkaline Media

Compound	Concentrations Moles/L	Approx. pH	Germination (%)* Evergreen Onion	American Flag Leek
NH_4OH	15	12.2	5.6	2.7
NH_2NH_2	15	11.7	23.0	8.0
CH_3NH_2	15	12.9	12.9	5.6
NaOH	0.01	12.0	58**	30*
	0.10	13.0	11	0
NH_4OOCCH_3	1	5.6	0	0
NaCl	1	6.0	0	0

* Minimum 100 seeds

** Green shoots after 7 days

Allium roots in 15 M ammonia were washed in distilled water until wash water was neutral or slightly acidic to indicator paper. Root sap was then pressed out and pH determined. Control roots were treated similarly. Roots in ammonia gave a pH of ca 8.5, whereas controls were at pH ca 6.5.

Allium roots in 15 M ammonia were washed and rapidly vacuum infiltrated with concentrated commercial Nessler Reagent, and washed again with vigorous shaking. Squash preparations were examined under oil-immersion at 1000X-magnification. A yellow-brown precipitate of the ammono-mercuric iodide complex was readily seen within many epidermal and cortical tissues closely following the shape of the cells. Many cells showed no precipitation, hence the overall effect was that of a mosaic pattern. Intercellular deposits were also in evidence.

Ammonia solutions lysed some membranes, such as those of beet root, rapidly and irreversibly transformed the betacyanin released to a yellow pigment. Beet root cubes 15 mm/side were immersed in ammonia solutions of 15 M, 1.5 M and 0.15 M for 90 min, then sectioned free-hand. Penetration was noted as follows:

Ammonia M	Penetration mm.
15	7-8
1.5	2-2.5
0.15	< 0.25

We realize that although ammonia tolerance in Allium may be unique to the genus, or to its family (Liliaceae), this uniqueness is unprovable by any effort short of testing sizeable populations of many varieties of many species in a large selection of genera and families.

In any case, the juxtaposition of contrasting individual ammonia tolerances in populations that must otherwise be highly similar offers a unique opportunity to study physiological performance under stress. No Allium radicle produced in strongly ammoniacal media can be considered "normal" for the cells produced are of a highly abnormal character. The attenuated state of the chromosomes in NH_3-grown roots may be a general stress response rather than a specific indicator of ammonia toxicity, because it resembles strikingly the condition of onion root tips frozen while growing, "... to give nucleic acid starvation of heterochromatic segments, which thus show their spiral structure".[1]

It is conceivable that ammonia intoxication interferes in some specific way with DNA synthesis. However, what little is known about the action of NH_3 or NH_4^+ on biochemical pathways suggest their interference in protein and amino acid metabolism (inhibition of some l-amino oxidases and glutamic-alanine transaminase). Presumably ammonia applied at the levels used here could also interfere with any process requiring Cu^{++}, Co^{++}, or Zn^{++}, all of which form ammono-complexes readily. It is evident that our present knowledge of ammonia as a metabolic inhibitor or regulator is highly limited.

To what extent can the effects of high levels of NH_3 be accounted for in terms of alkalinity or osmolarity? In NaOH (pH 12) the high germination rate and subsequent growth into normally differentiated green seedlings suggests that Allium is even more remarkably tolerant of alkalinity than it is of NH_3. At pH 13, NaOH still supports more germination than NH_3, in the onion, but not in the leek variety tested. Furthermore, methylamine at pH 12.9 supports somewhat higher germination than NH_3 at pH 12.2. Osmotic or saline inhibition

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1. Darlington, C. D. and LaCour, L. F., "The Handling of Chromosomes" 3rd Edition, p. 91, G. Allen and Unwin Ltd., London, 1960.

is illustrated, presumably, by the effect of molar NaCl (ca 6%). However, even though aqueous NH_3 is far less dissociated than molar NaCl, its osmotic pressure at 15 M is still greater by tenfold than the salt solutions. Conceivably, NH_3, NH_4^+ or OH^- in ammonia solutions offsets the inhibitory activity which such solutions should exert on purely osmotic grounds, just as Ca^{++} offsets inhibition of rye germination by NaCl. These comparisons render interpretation of the inhibitory effects of ammonium acetate uncertain.

III. ADDITIONAL DATA: RESPONSES TO NH_3 AND ALKALIS

A. *Allium* Germination in Strong Alkali

Unlike NH_3, which supported the germination of about 75% of 30 varieties and allows only 10% germination at most, 0.01 M Na or KOH at the same pH (12) permitted at least minimal germination in all varieties. Germination in the 50-80% range were fairly commonplace in the alkalies (Table IX), as were green shoots.

The Na^+-K^+ differential is quite striking in some varieties and not evident in others. Thus, 8 out of 26 varieties showed greater germination in KOH than in NaOH; 5 varieties responded better in NaOH; and 13 were marginally affected or indifferent.

The ammonia effect must be in part a result of the presence of NH_4^+ and OH^-; hence it was of interest to look for signs of correlation of *Allium* responses to various ammoniacal and alkaline conditions at 1 atmosphere. The four sets of conditions selected were (Figure 1):

1:1 NH_3:air vs 0.01 M NaOH

1:1 NH_3:N_2 vs 0.01 M NaOH

1:1 NH_3:N_2 vs 0.01 M KOH

1:1 NH_3:air vs 0.01 M KOH

Graphically no strong definite trends appeared, but the best correlations appear to reside in aerobic ammonia cultures, especially against KOH.

The data show that among the stronger bases, the mechanisms in operation are cation dependent in about one-half of the varieties used.

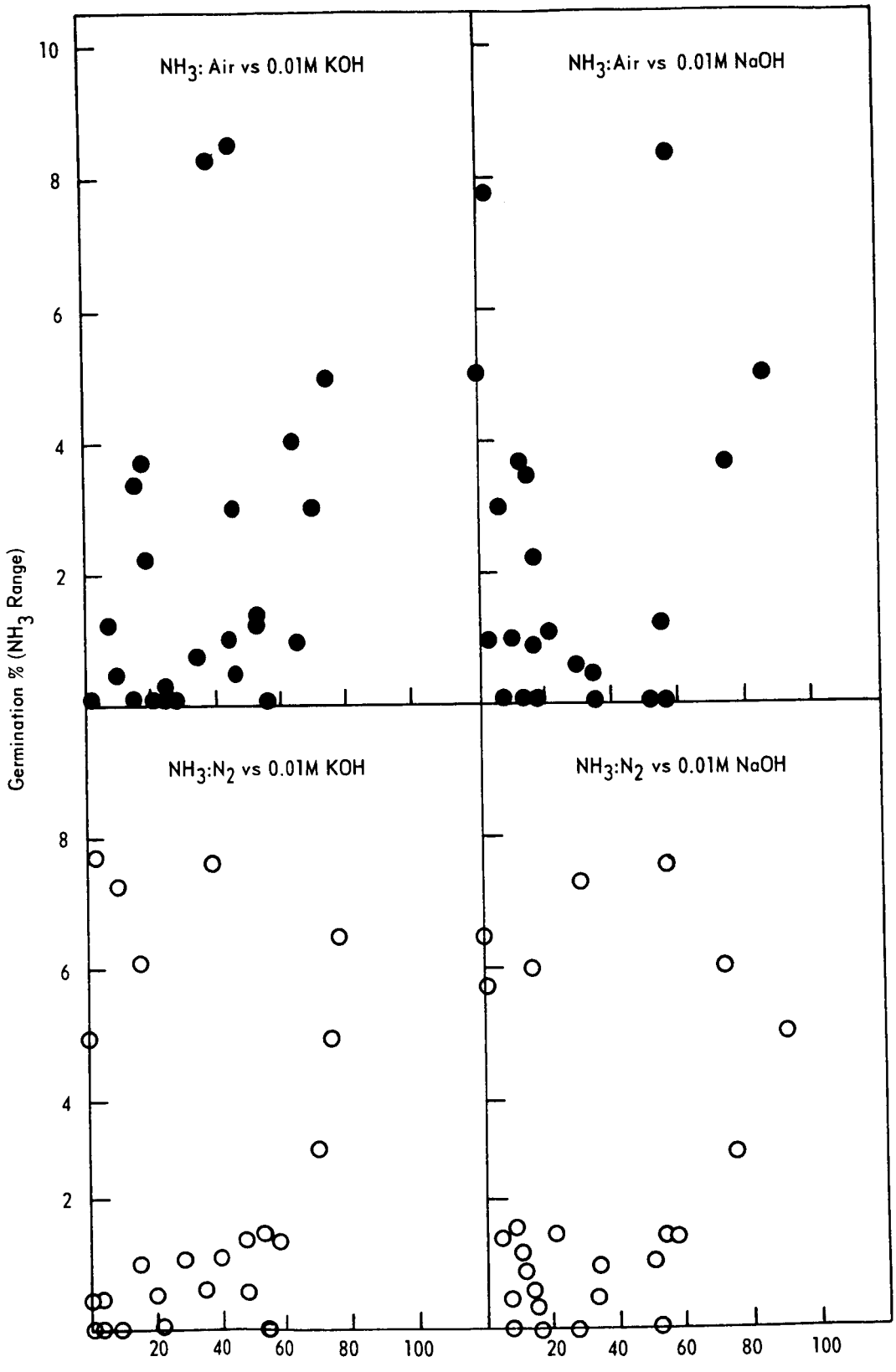


FIGURE 1 CORRELATION TESTS BETWEEN GERMINATION IN AMMONIACAL AND ALKALI MEDIA

Table IX Germination of Allium Cultivars at pH 12

in 0.01 M Alkali

	% Germination 4 Days	
	.01 M KOH	.01 M NaOH
Onion		
Yellow Globe Danvers	54	54.3
White Portugal	47.5	6.5
Crystal White Wax	1.5	9.0
Downing Yellow Globe	14.5	34.0
Ebenezer	47.5	33.0
Burpee Yellow Globe Hybrid	35.0	17.5
Early Yellow Globe	56.5	56.5
Southport Yellow Globe	44.0	9.5
Southport Red Globe	2.0	15.5
Southport White Globe	24.5	51.5
Red Wetherfield	9.5	29.5
Early Grano	64.0	72.0
Autumn Spice Hybrid	27.0	12.5
Excel Bermuda	27.0	18.0
Australian Brown	8.0	2.5
Yellow Bermuda	21.0	9.5
Sweet Spanish	17.0	14.5
Sweet Spanish Hybrid	53.0	52.5
White Sweet Spanish	75.0	86.0
He Shi Ko	69.5	77.0
Magnifero Hybrid	68.5	21.0
White Lisbon	18.0	12.5
Evergreen Long Bunching	39.0	58.0
Leek		
Elephant	59.0	1.5
Large Musselburg	78.0	0
Broad London	23.0	30.0

The suppression of green shoots in ammonia at pH 12 is not a result of elevated pH as the stronger bases show. However, if the stronger bases are applied at higher concentrations, germination is once again reduced to root emergence during the four day period.

		Molarity					
		KOH			NaOH		
		0.01	0.1	1.0	0.01	0.1	1.0
Onion (Evergreen)	Germination	39	39	48	58	11	23
	Green shoots	+	-	-	++	-	-
Leek (Broad London)	Germination	23	2	17	30	0	9
	Green shoots	+	-	-	++	-	-

The possible uniqueness of ammonia tolerance among seed plants has been noted in Section II. Additional data can now be added to amplify this point.

Family Liliaceae	Germination (%) in NH_4OH (Concentrated)
Genus	
Asparagus	2.2 (dead by day 4)
Lilium (regale)	0
Hemerocallis	0
Ornithogalum	0
Allium (chives)	4.3

Asparagus showed an abortive response, three other genera none, and only one other member of Allium shows essentially a typical ammonia response.

B. Some NH_3-Induced Histochemical Changes in Xerophytic Forms

In an effort to extend our understanding of the changes that exposure to NH_3 might effect in vivo, entire plants of Cereus peruvianus and several Euphorbias were held in a 1:1 NH_3:Air composition for ca 4 weeks. Grossly, plants were bronzed, but had not yet collapsed, or become extended "culture media" for tolerant microflora. Tissues were somewhat flaccid, but not generally shrivelled. One species E. xylophylloides had turned to a glossy deep black color on the surface, but was otherwise intact.

Respiration measurements were attempted on three specimens: Cereus, E. hermentiana, and E. xylophylloides. No CO_2 production could be detected, but the tissues, still moist and NH_3-laden, could have retained CO_2 as HCO_3^- or $CO_3^=$.

O_2 uptake in air on a "per plant" basis was as follows (cm^{-3}/hr):

	Treatment	
	In NH_3	Control
E. hermentiana	10	6
E. xylophylloides	6	4
Cereus	6	4

Plants exposed to NH_3 showed a characteristically elevated O_2 consumption, either a result of increased respiratory substrates, autoxidizable reducing agents, or increased permeability. In view of a) the comments on ammonia penetration in II (p. 17), b) the continued exudation of ammonia from xerophytic tissue for 2 days, and c) the generally moist surface of the specimens, the permeability factor was considered to be a primary factor in enhanced O_2 consumption. Furthermore, unlike untreated plants, those out of NH_3 shrivelled within a few days in air.

Histochemical tests (Table X) showed that irrespective of species, starch aldehydes and o-diphenols, when present at all, were moderately to greatly elevated after NH_3 treatment. Levels of enzymes are difficult to explain at present except for the frequent reciprocal peroxidase-catalase relation.

Table X Histochemical Comparisons

		Starch	Aldehyde	Ortho-diphenols	Dehydrogenase	Catalase	Peroxidase
Euphorbia							
hermentiana	NH_3	±	++	++	+	+	++
	Control	±	±	+	+	++	+
obesa	NH_3	-	+	±	-	-	++
	Control	-	-	-	-	++	+
sasone	NH_3	±	++	+	+	+	++
	Control	-	+	-	+	++	+
medusa	NH_3	+	+++	-	±	+	+
	Control	-	-	-	±	+	+
xylophylloides	NH_3	+	+++	±	±	+	+
	Control	±	±	-	±	+	+
Cereus	NH_3	++	++	±	±	+	+
	Control	+	-	-	-	+	+

IV. THE MICROBIOLOGY OF ULTRASALINE, ACIDIC, AND OTHER EXTREME CHEMICAL ENVIRONMENTS

A. Review of Preliminary Experiments

In the last report (Semiann. Rpt., 1 Nov. 1965) data were given showing the successful culture of resistant or tolerant strains of bacteria from soil inocula in extreme chemical environments, and the maintenance of viable, motile cells in such cultures for 6 months.

The status of these crude exploratory media from inception is as follows:

Saturated Salt Media with Motile Bacteria

At 1 Month	6 Months	1 Year
$LiCl$, $LiBr$, $LiNO_3$	$LiCl$, $LiBr$	$LiCl$
$NaCl$, Na_2SO_4, Na_2HPO_4, Na_2S, Na_2SO_3	All active	$NaCl$, Na_2SO_4, Na_2HPO_4
KBr, KNO_3	Both active	KBr
$MgCl_2$, $MgSO_4$	Both active	$MgCl_2$
$CaCl_2$	Active	Active
$Sr(NO_3)_2$	Active	No activity
$BaCl_2$	Active	Active
NH_4OAc, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $(NH_4)_2CO_3$	All active	NH_4Cl, $(NH_4)_2PO_4$

Failures may have involved substrate or metabolite depletion, breakdown in resistance factors, toxic chemical changes in media, etc. Nevertheless, the performance is striking. Among heavy metal media at one year, viable cells

still exist in Cu(II), Co(II), Mu(II) and Fe(III) salts, except nitrates.

Other special cultures in alkaline media show at one year:

a. KCN - motile bacteria present, and odor of NH_3 from soil cultures strong, solution saturated;

b. NaOH - no bacteria surviving in 1 N media;

c. H_2SO_4 - no bacteria surviving at any level above 1 N, except for suspected spore germination in sediment in 36 N acid.

It will be recalled that preliminary efforts in the use of defined media were also described in the 1 Nov. report. A comparison of the 3 and 9 month data shows that during the past 6 months some have increased, some have decreased and others have remained essentially unchanged in bacterial count, (Table XI).

B. Current Results

In the earlier phases of our study of ultrasaline media, an accidental laboratory contaminant was found in certain cultural materials; for example, in preservative-free bakery bread saturated with KCl, Na_2SO_4, $Ca(OAc)_2$, etc. This contaminant, a fungus, did not appear until some of these cultures had been maintained for periods of at least 12 weeks, and, in some instances, not until almost a year had elapsed. Once established in saline-bread medium, this contaminant could readily and rapidly be transferred to many other media and environments without a perceptible delay in growth response. It appears that we encountered a mutant clone of a common laboratory contaminant, Penicillium notatum, which mutant now possessed an astonishingly wide range of stress tolerance. Morphologically, P. notatum (clone "extremis") appears to be indistinguishable from the wild type when grown in conventional culture media such as Sabaroud's agar, Czapek's agar,

Table XI Review of Soil Bacterial Growth in Defined Media After 3 and 8 Months

Growth of Bacteria at 22°C in Defined Salt Media From Soil Inocula (5g/ℓ)

Salt Saturated	Medium[a]	Observations after 90 Days Count[b]	After 270 Days Count	Δ 270-90
		N X 10^6	N x 10^6	
LiCl	YG	0.2	3.5	+
	PG	3.9	3.0	0
	YE	> 10	3.5	-
	GE	10	2	-
$MgCl_2$	YG	0.8	0.5	0
	PG	2.1	2.0	0
	YE	5.1	3.0	-
	GE	1.0	0.5	-
$CaCl_2$	YG	0.8	0.5	0
	PG	7.0	2.0	-
	YE	1.1	2.0	+
	GE	0.8	2.5	+

[a] YG=5g/ℓ yeast extract + 10g/ℓ glucose; PG=5g/ℓ peptone, 10g/ℓ glucose; YE=5g/ℓ yeast extr. in Eagles med.; GE=10g/ℓ glucose in Eagles med.

[b] Initial cell count < $10^4/cm^3$ (< 0.01 x $10^6/cm^3$).

nutrient broth, etc., at temperatures ca 22°C. On some specific salt media, for example $Mg(OAc)_2$, the mycelial mats retained the color and general appearance of P. notatum. However, in practically all media containing saturating levels of salts, definite changes in spore color, mycelial appearance, color and fluorescence of broth medium, and cytology have taken place. To illustrate:

KCl – mycelial mats indented, distinctly margined, spores brown, medium colorless;

NaOAc – mycelium normal but underside of mycelial mat carries spheroidal masses of $NaHC_2O_4$, spores olive brown, medium pale yellow;

$Mg(OAc)_2$ – mycelium normal, spores bright green, medium brilliant yellow and contained copious chalky precipitate of $MgCO_3$;

$SrCl_2$ – mycelium composed of loose spherical subsurface bodies, spores rare, black giving salt and pepper effect against white mycelium, medium colorless;

H_3PO_4 – 1N mycelium white, loosely margined mat with hyphal masses pendant in medium, spores few and pale, medium colorless;

4N mycelium large, loose spherical subsurface body, spores few and pale, medium colorless.

These comments also apply to unusual media other than salts. The ability of an organism to make use of the ion-coordinated water in a saturated solution was not the only factor of interest in these experiments. Superimposed on the salinity factor was the temperature variable (Table XII). Spore-inoculated salt cultures were incubated under each of three

Table XII Interaction of Temperature and Salinity on *Penicillium* Growth (in Standard Peptone-Yeast-Glucose Media)

Saturated Salt Solution	A_w* (ca 20-30°C)	First Observation of Macroscopic Mycelia (weeks) 4°C	22°C	16 hr-30°C 8 hr + 22°C Cycle
Chlorides				
NH_4	0.78	-	24	-
Li	0.11	-	24	-
Na	0.76	-	24	-
K	0.84	2	1	2
Mg	0.33	-	1	5
Ca	0.30	-	8	-
Sr	---	2	1	4
Ba	---	-	1	-
Control	1.	2	2/7	2
Acetates				
NH_4		-	-	-
Li	0.11	-	-	-
Na	0.73	2	1	4
K	0.23	-	-	-
Mg	0.55	2	1	2
Ca	0.85	2	1	2
Sr	---	-	4	-
Ba	---	-	8	-
Control	1.	2	2/7	2

* $A_w = \frac{\text{aqueous vapor pressure of salt solution}}{\text{vapor pressure of pure water}}$ at T°C.

different regimes. The first group was kept at room temperature (22°C), the second at a constant 4°C, and the third was placed on a cycle of 16 hours per day at -30°C and 8 hours per day at 22°C. Eight culture flasks of each inoculated saturated salt solution were incubated at each temperature in order that samples might be drawn from the supply at designated intervals. Observations and comparisons of the data obtained from these samples showed a complex interaction of the salt and temperature variables. Not only were there obvious differences in the growth curves of cultures incubated in different salt solutions (Figure 2), but the growth curves of the organism incubated in the same salts at several temperature levels showed marked variation (Figures 2-4). The media contained 0.1% peptone, 0.1% yeast extract, and 0.5% glucose.

Large differences in the extent of growth in saturated salt solutions led us to experiment with combinations of salts. Since it had been previously noted that divalent salts were, in general, less inhibitory to growth than monovalent salts, combinations of these two types of salts were prepared. Results (Table XIII) showed that the effect was a complex one. When LiCl, normally a growth inhibitor, was combined with non-inhibitory $MgCl_2$, the solution supported growth. However, when LiCl was combined with non-inhibitory $CaCl_2$, the resultant solution did not support growth. Other results of salt combination are also possible: for example, the acetates of Na and Sr, when combined, no longer supported growth.

While a liquid medium was found to be best for P. notatum (clone "extremis"), a solid medium distinct from agar in physical properties was tested. This medium was an electron-beam-radiated cross-linked polyethylene oxide gel which was loaded with saturated solutions of the experimental mineral salts. The advantage of the "Polyox" gel was

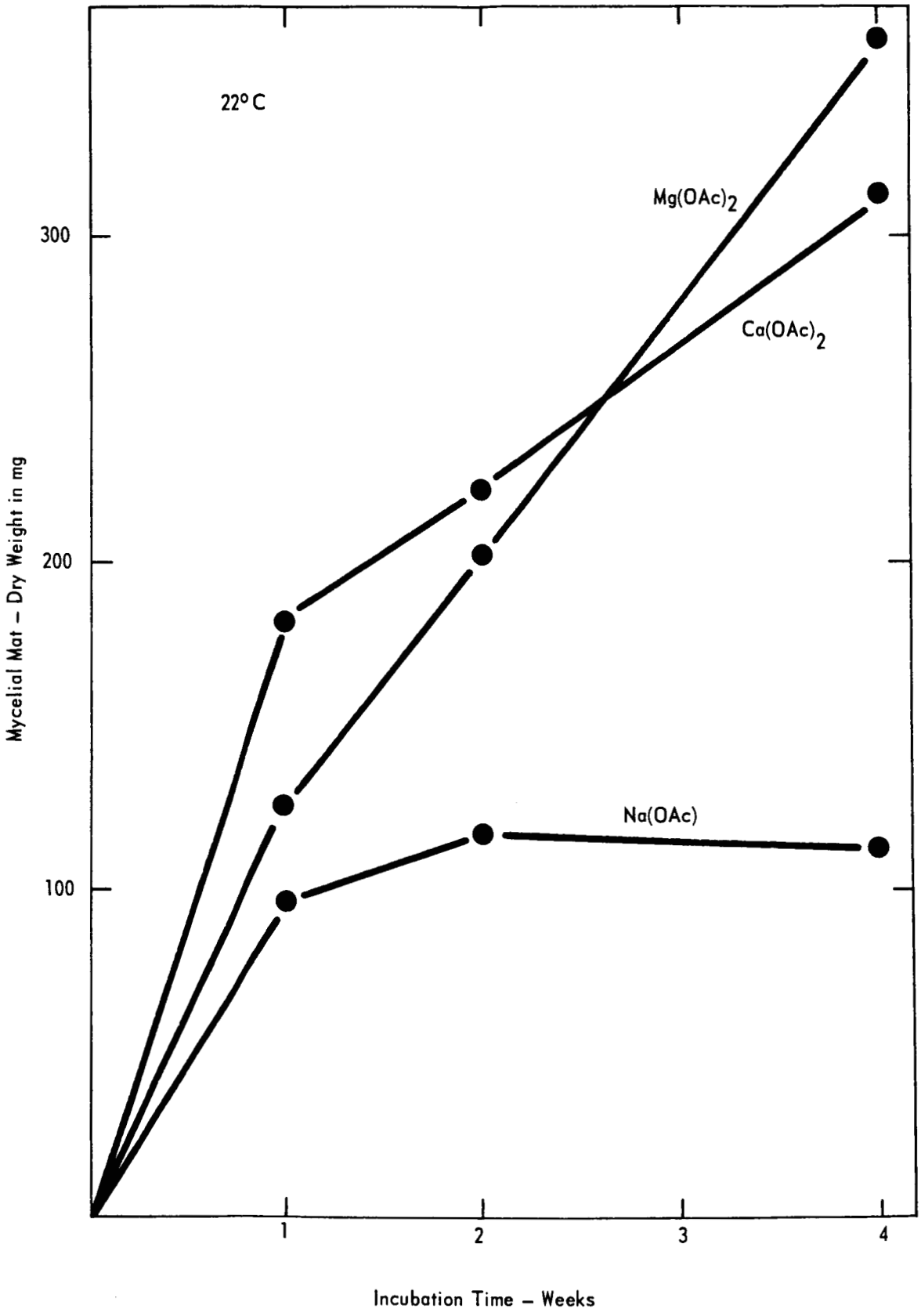


FIGURE 2 THE COURSE OF GROWTH OF P. NOTATUM IN SATURATED ACETATE SALTS (50 ml)

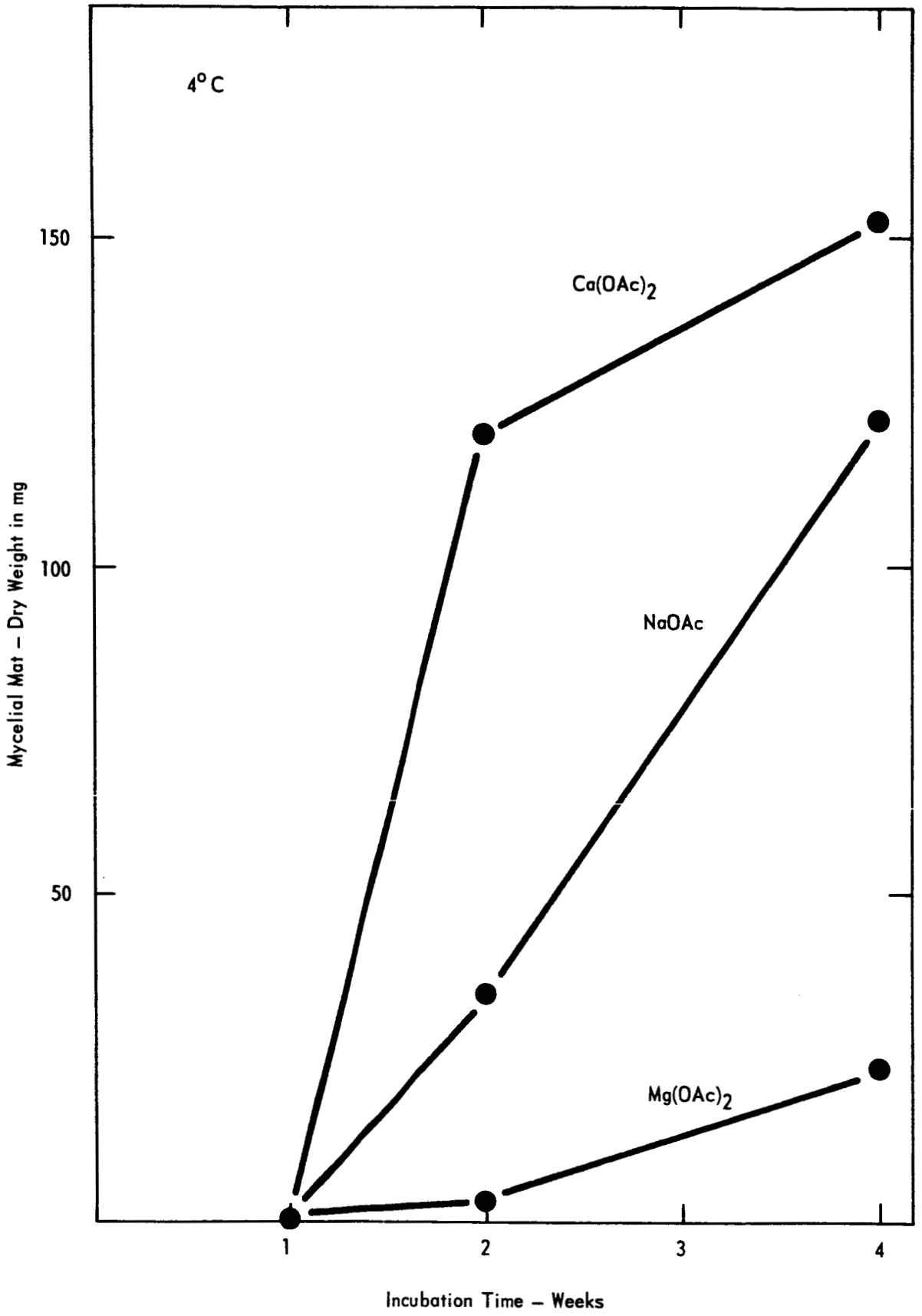


FIGURE 3 THE COURSE OF GROWTH OF P. NOTATUM IN SATURATED ACETATE SALTS (50 ml)

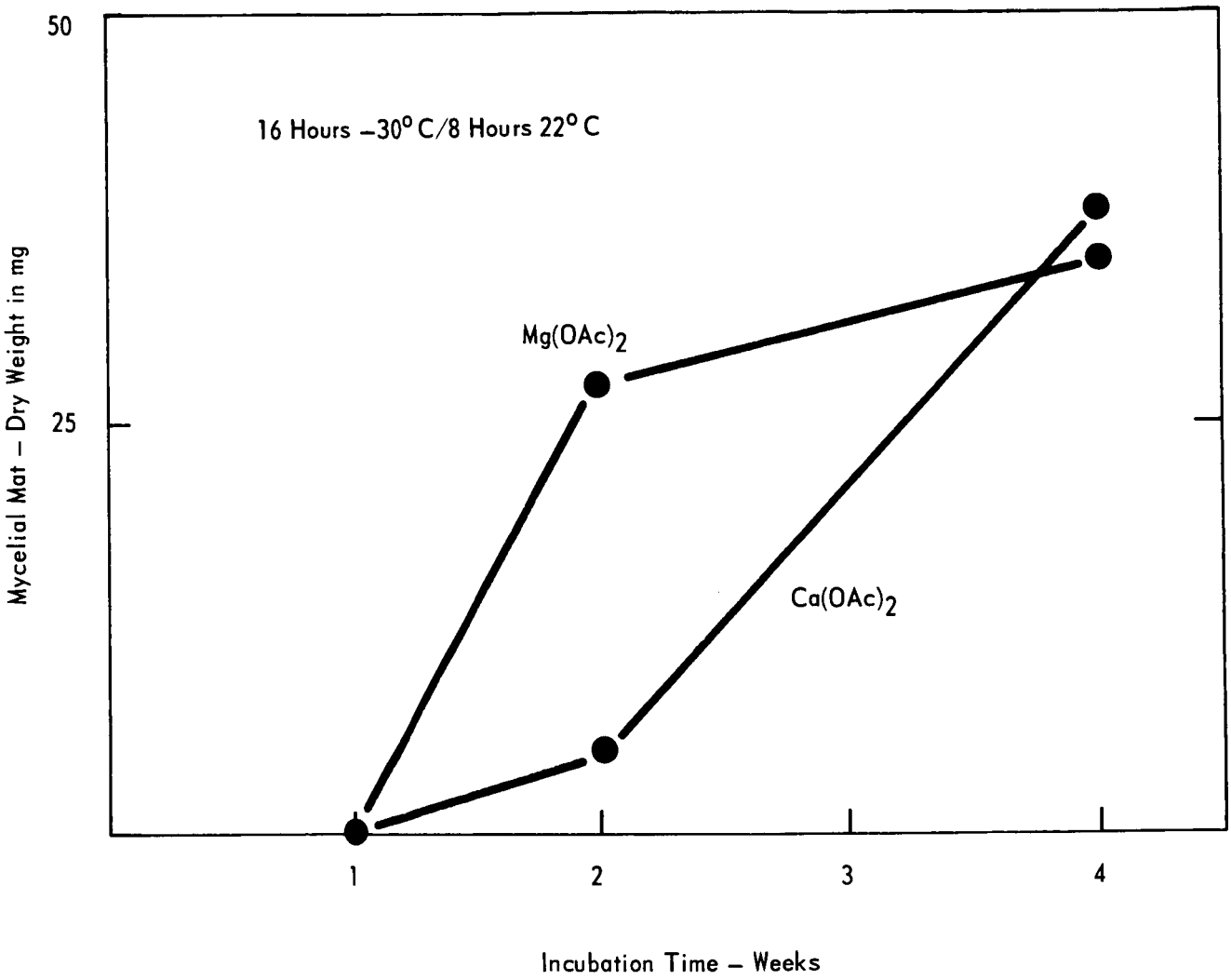


FIGURE 4 THE COURSE OF GROWTH OF P. NOTATUM IN SATURATED ACETATE SALTS (50 ml)

Table XIII Growth of *Penicillium* in Combinations of Saturated Salts

Salt		Macroscopic Mycelial Mat after 8 wks at 22°C		
a	b	a only	b only	a + b
LiCl	$SrCl_2$	0	+	0
LiCl	$MgCl_2$	0	+	+
LiCl	$CaCl_2$	0	+	0
NaCl	$SrCl_2$	0	+	+
NaCl	$MgCl_2$	0	+	+
NaCl	$CaCl_2$	0	+	+
KCl	$SrCl_2$	+	+	+
KCl	$MgCl_2$	+	+	+
KCl	$CaCl_2$	+	+	0
LiOAc	$Sr(OAc)_2$	0	+	0
LiOAc	$Mg(OAc)_2$	0	+	0
LiOAc	$Ca(OAc)_2$	0	+	0
NaOAc	$Sr(OAc)_2$	+	+	0
NaOAc	$Mg(OAc)_2$	+	+	+
NaOAc	$Ca(OAc)_2$	+	+	0

that it not only permitted a separation of discrete colonies but also allowed the extension of individual hyphal tips or masses of hyphal tips to be followed directly at high dry magnification (ca 450 x). The results of tests on this unique medium are shown in Table XIV.

Once a sufficient supply of salt-tolerant molds had been accumulated, it became possible for us to begin tests which would reveal biochemical differences among the various P. notatum cultures. Table XV shows some preliminary results in this area. Significant differences were observed not only in enzyme activity, but also in total nitrogen content. Further studies on the physiological functioning of these molds are in progress.

The degree of adaptation shown by our clone of P. notatum to high salt concentrations led us to an investigation of the organism's ability to adapt itself to other harsh chemical environments. A listing of the compounds chosen for this study is given in Tables XVI-XVIII. Many of these cultures have already yielded promising results, although their period of incubation has been short. Continued observation may give us further insight into the nature of survival under extreme stress.

Table XIV Growth of Penicillium in Saturated Salt Solution Media[a] and on "Polyox"[b] Treated with These Solutions

Salt	Form of Growth	Growth Rate Based Upon Linear Extension of Hyphae at 22°C after 144 hr, μ/24 hr	Growth After 144 hr in Saturated Liquid Medium
LiCl	Many isolated hyphae	< 50	0
LiAOc	as LiCl	100-200	0
NaCl	as LiCl	100	0
NaOAc	20 colonies 1-5 mm	400-750	+
KCl	as LiCl	< 50	+
KOAc	Entire plate a hyphal mass	300-700	-
$MgCl_2$	as LiCl	50-100	+
$Mg(OAc)_2$	as LiCl	50-100	+
$CaCl_2$	as LiCl	50-100	-
$Ca(OAc)_2$	> 40 colonies 1-10 mm	> 1000	+
$SrCl_2$	0.5-1	100	+
$Sr(OAc)_2$	21 colonies 2-4 mm	350-700	-

[a] Contains 0.1% peptone, 0.1% yeast extract, 0.5% glucose;

[b] 2% cross linked polyethylene oxide in 9 cm polystyrene petri dishes.

Table XV A Partial Compilation of Growth and Metabolic Data for Penicillium[a] on Saturated Salt Media.[b]

	Acetate				Chloride		Nutrient Control
	Na	Mg	Ca	Sr	K	Sr	
A_w	0.73	0.55	0.85	--	0.84	--	ca 1
Mycelial Dry wt (mg.)	140	602	540	--	70	41	98
N content (%)	2.22	2.22	0.34	0.80	1.39	1.43	3.53
Amylase Secretion Starch Agar (diam. of hydrolysis zone, mm)	-	6	1	--	0	0	11
Phosphorylase Secretion Glucose-1-Phosphate Agar (diam. of synthesis zone, mm)	-	6	7	--	13	12	20
Phenolase Activity (Purpurogallin color/20 mg/1 hr)	14	14	25	24	9	5	15
Peroxidase Activity (Purpurogallin color/20 mg/2 min)	29	15	25	25	20	7	15

[a] Cultures incubated at 4°C.

[b] Contains 0.1% peptone, 0.1% yeast extract, 0.5% glucose.

Table XVI Acid Test Media for Penicillium notatum

(8 wks)

Medium* in	Normality of Acid	Response of Conideospores: Swelling	Germin.	Micro-hyphae	Macro-Mycelium
H_2SO_4	1	+	+	0	0
	2	+	0	0	0
	4				
HCl	1	0	0	0	0
	2	0	0	0	0
	4	0	0	0	0
HNO_3	1	0	0	0	0
	2	0	0	0	0
	4	0	0	0	0
H_3PO_4	1	0	0	0	+ (heavy mat)
	2	0	0	0	+ (heavy mat)
	4	0	0	0	+ (submerged)
	10	0	0	0	0
CH_3COOH	1	0	+	+	0
	2	+	+	0	0
	4	+	+	0	0
	10	+	+	0	0
	17	0	0	0	0
H_2NSO_3H	1	+	0	0	0
	2	0	0	0	0
	ca 4	0	0	0	0

* Contains 0.1% peptone, 0.1% yeast extract, 0.5% glucose.

Table XVII Basic Test Media for Penicillium notatum

(8 wks)

Medium* in	Normality of Base	Growth of P. notatum	Other Observations
KOH	1	0	ca 10^3 micrococci/ml
	2	0	rare motile bacteria
	4	0	
	10	0	
NaOH	1	0	ca 10^1 micrococci/ml
	2	0	
	4	0	
	10	0	
CH_3NH_2	1	0	
	2	0	
	4	0	
	6	0	

* Contains 0.1% peptone, 0.1% yeast extract, 0.5% glucose.

Table XVIII Other Test Media Under Observation for

Penicillium notatum

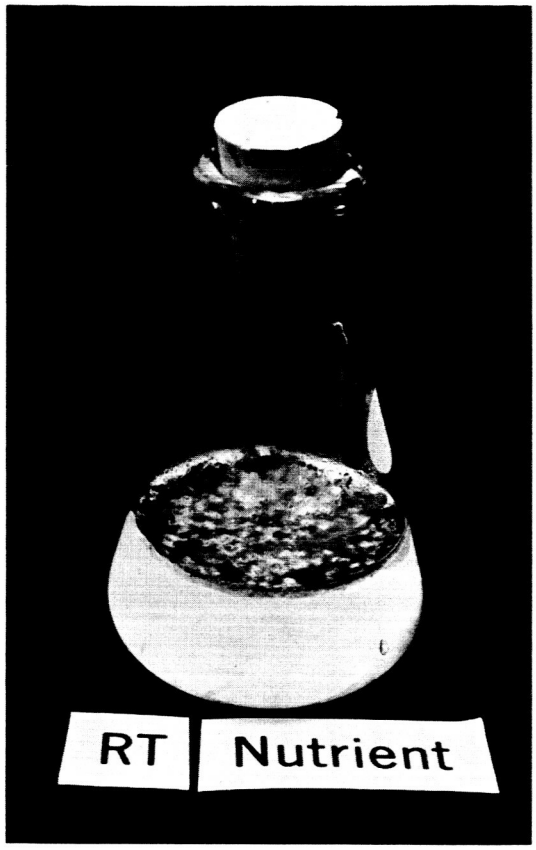
Medium*	Temperature	Observations (4 wks)
CH_3OH, Aqueous		
Vol. % 100	22°C	No growth
	-40	No growth
75	22	Spores germinating (rare)
	-40	Spores swell
50	22	More numerous germination, many swell
	-40	Rare spore germination
NaF, Aqueous		
Saturated	22	Spores germinating
	50	Spores swollen greatly, no germ.
	78	Spores swollen
	104	No change noted
Glycerol	22	Spores germinating
	50	Spores germ. rare
	78	Spores swell
	104	No changes noted

* Contains 0.1% peptone, 0.1% yeast extract, 0.5% glucose.

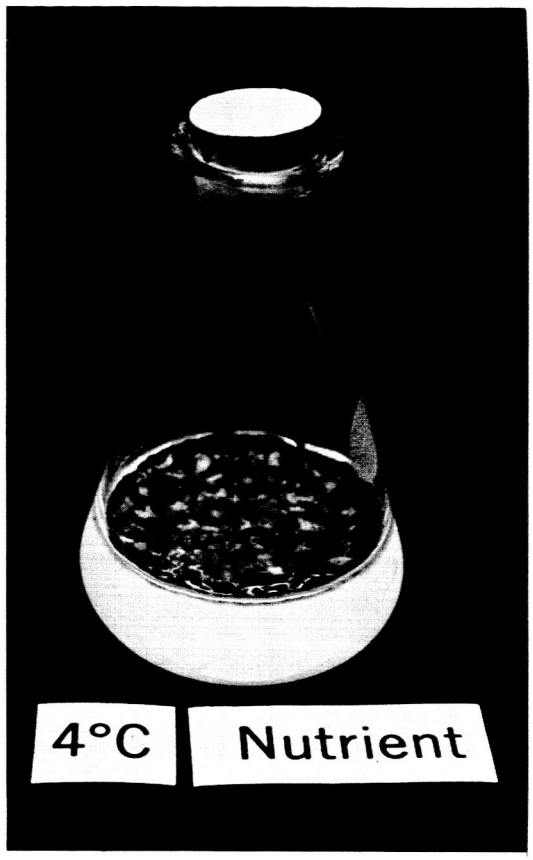
PENICILLIUM NOTATUM

UCRI CLONE "EXTREMIS"

Photographs Compare the Responses to Different Saturated Salt Solutions at Different Temperatures



A



B

C

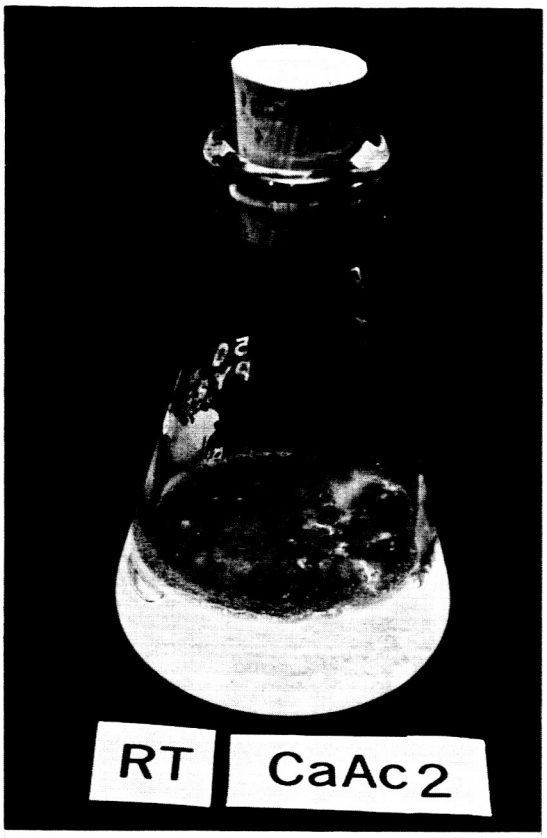
Nutrient Broth Culture at:

A) 22°

B) 4°

C) 16 hours at –30°
8 hours at 22°

Plate 1



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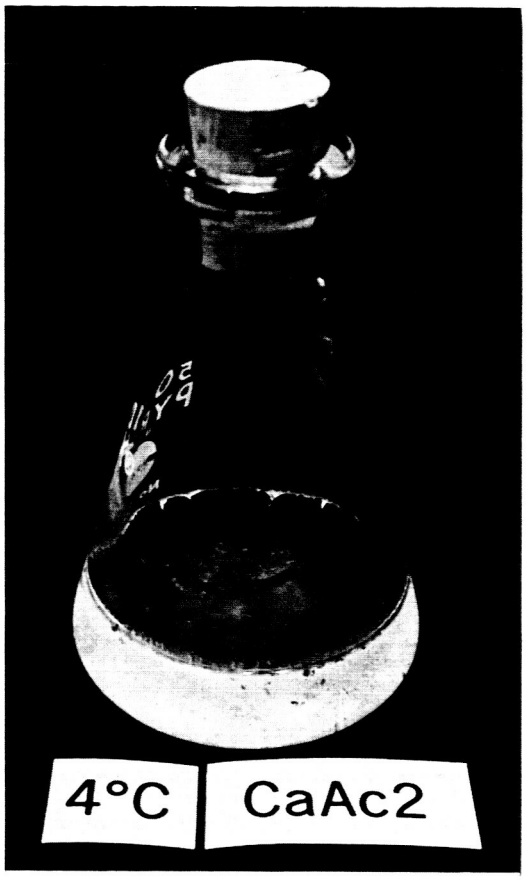
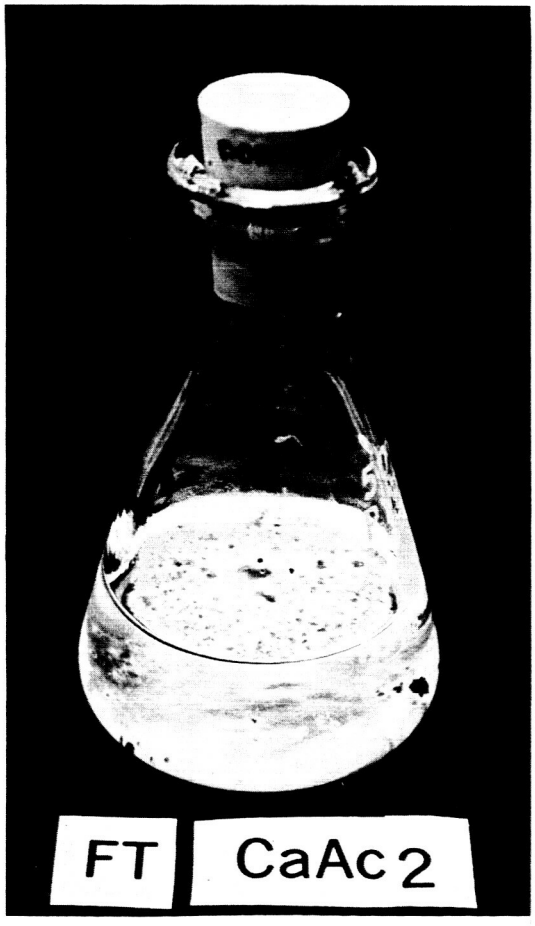


B

C



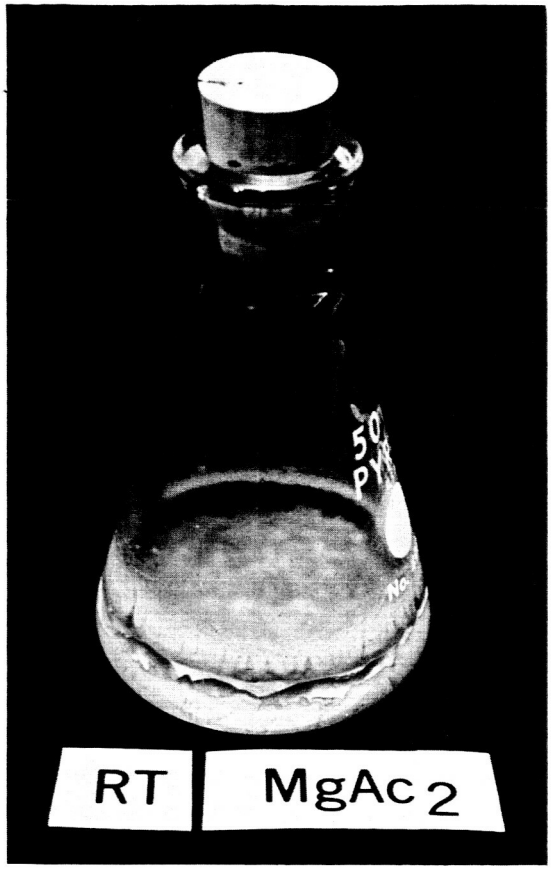
Saturated $Ca(OAc)_2$ Broth Culture at:

A) 22°

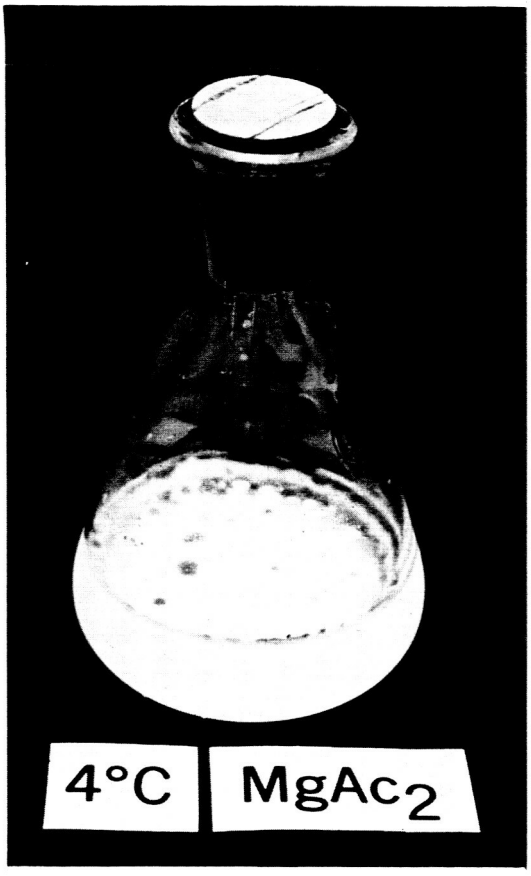
B) 4°

C) 16 hours at −30°
8 hours at 22°

Plate 2



A



B

C

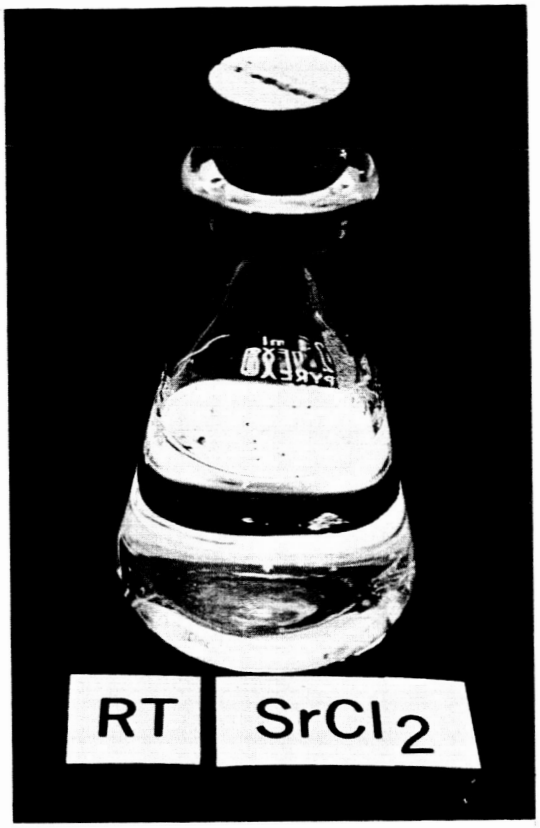
Saturated $Mg(OAc)_2$ Broth Culture at:

A) 22°

B) 4°

C) 16 hours at –30°
8 hours at 22°

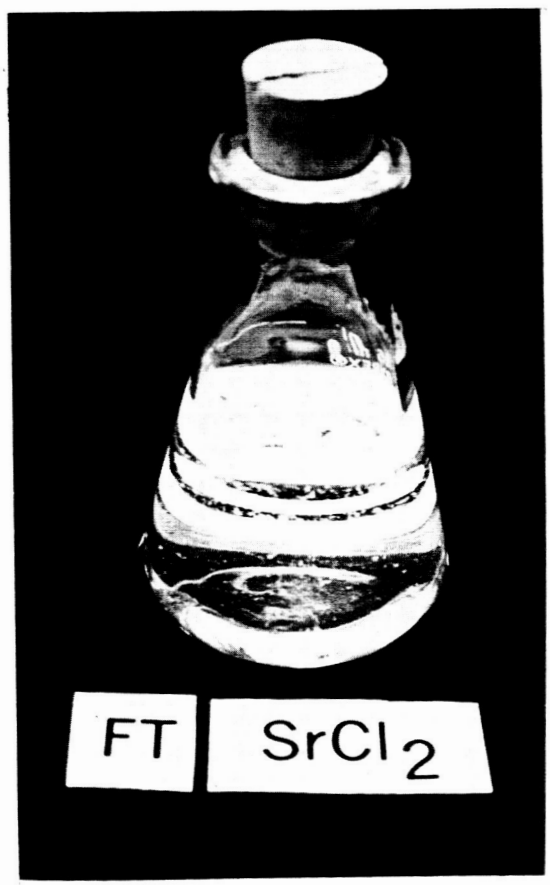
Plate 3



A

B

C



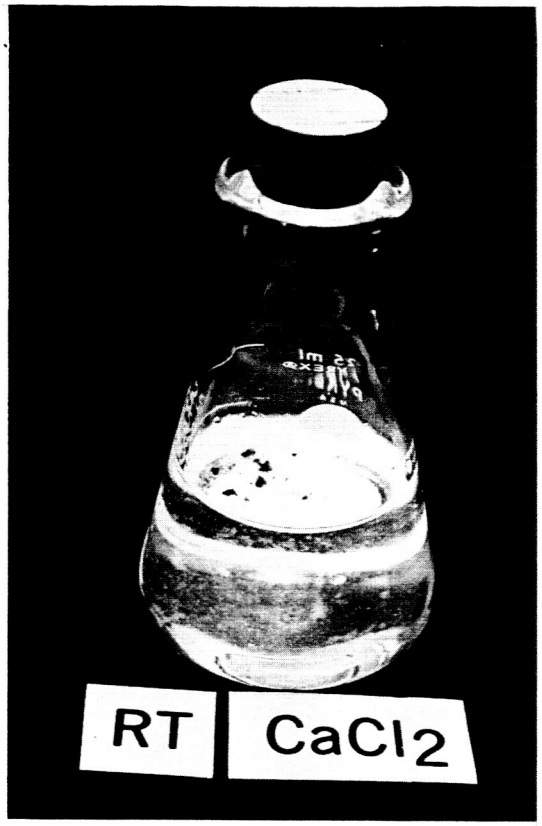
Saturated $SrCl_2$ Broth Culture at:

A) 22°

B) 4°

C) 16 hours at –30°
8 hours at 22°

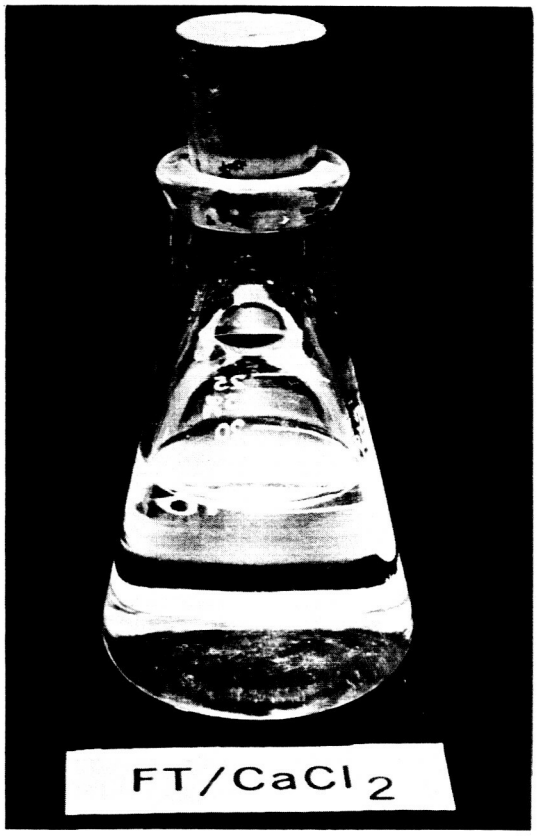
Plate 4



A

B

C



Saturated $CaCl_2$ Broth Cultures at:

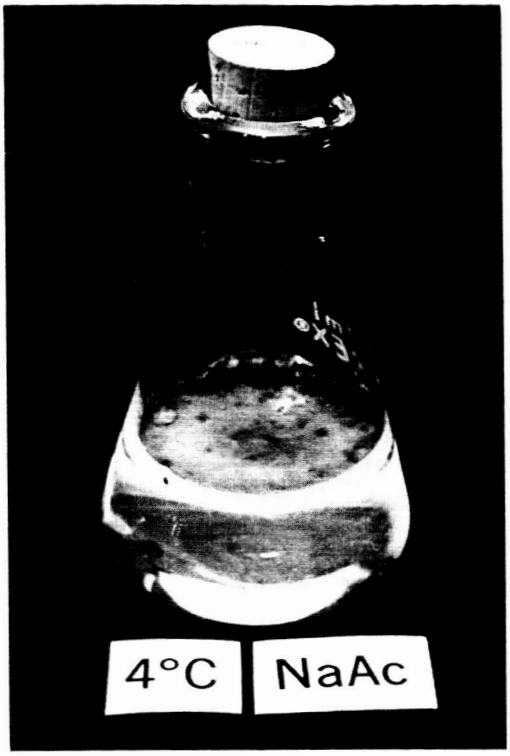
A) 22°

B) 4°

C) 16 hours at −30°
8 hours at 22°

Plate 5

A

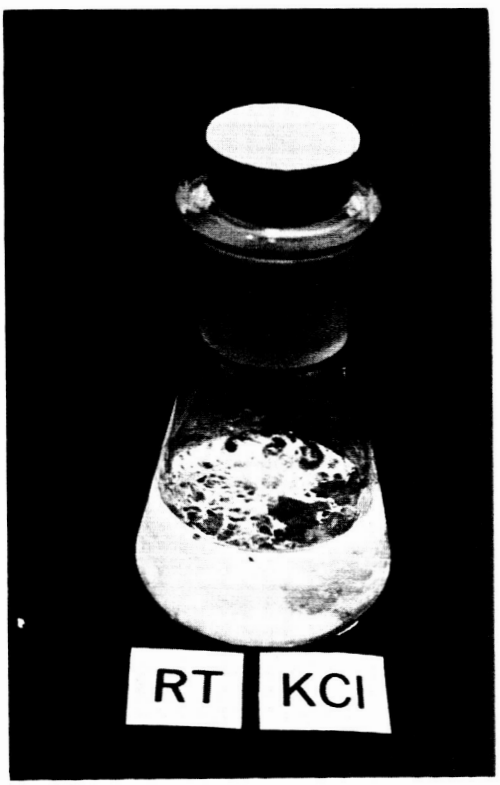


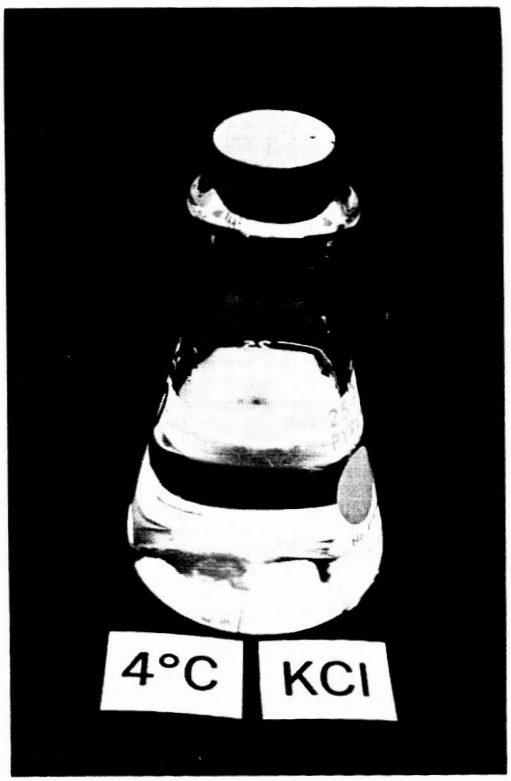
B

Top: Saturated NaOAc Broth Cultures Incubated at 22° (left) and at 4° (right).

Bottom: Saturated KCl Broth Cultures Incubated at 22° (left) and 4° (right).

C

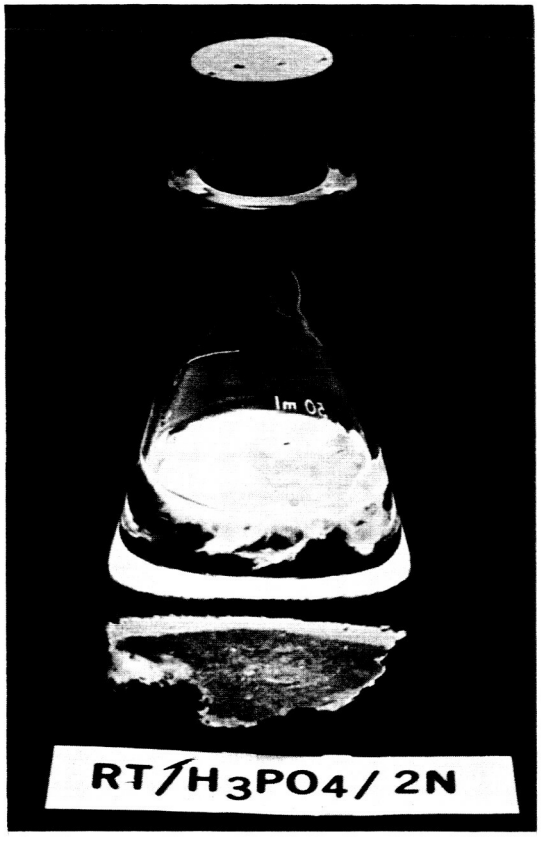




D

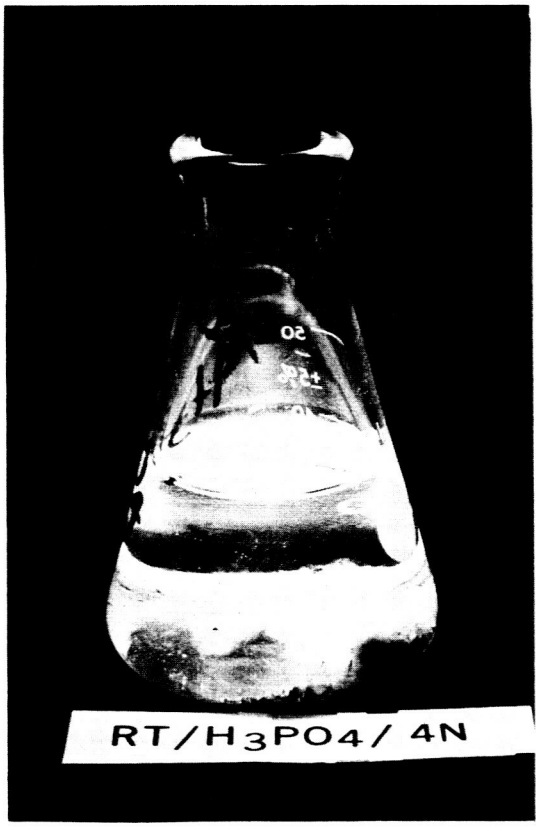
Plate 6

A



B

C



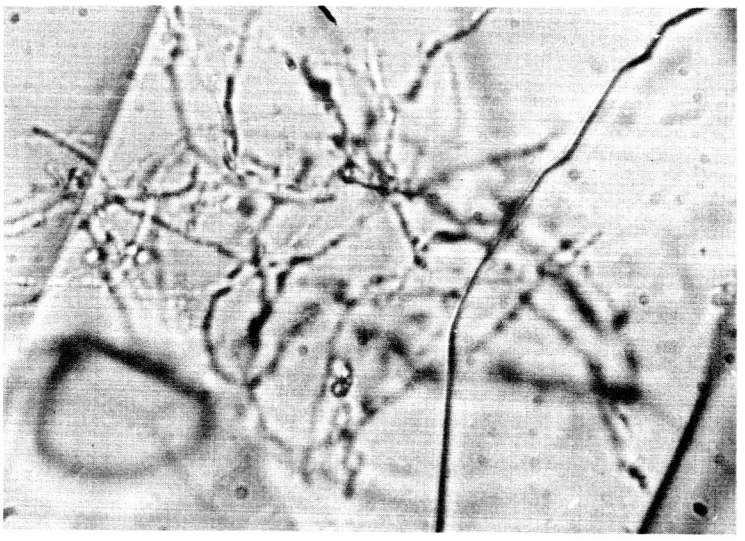
Growth of Penicillium Notatum at Room Temperature in:

A) 1N H_3PO_4
B) 2N H_3PO_4
C) 4N H_3PO_4

Plate 7

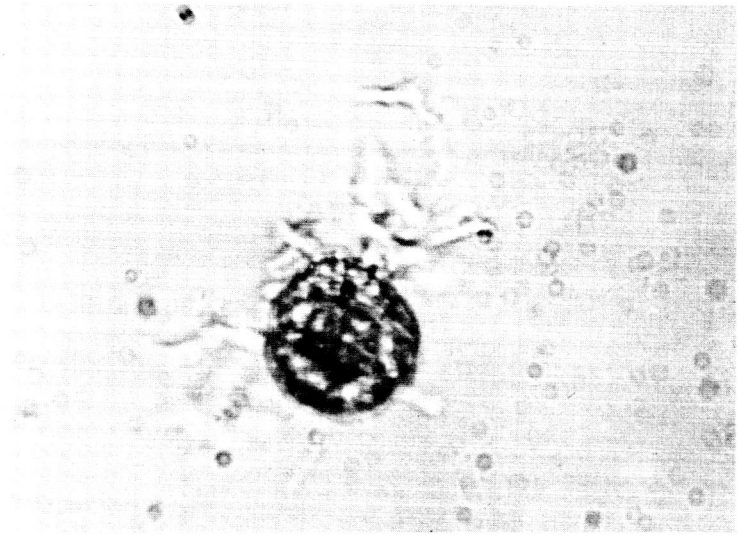
Photomicrographs of Mold

Growth in Various Media



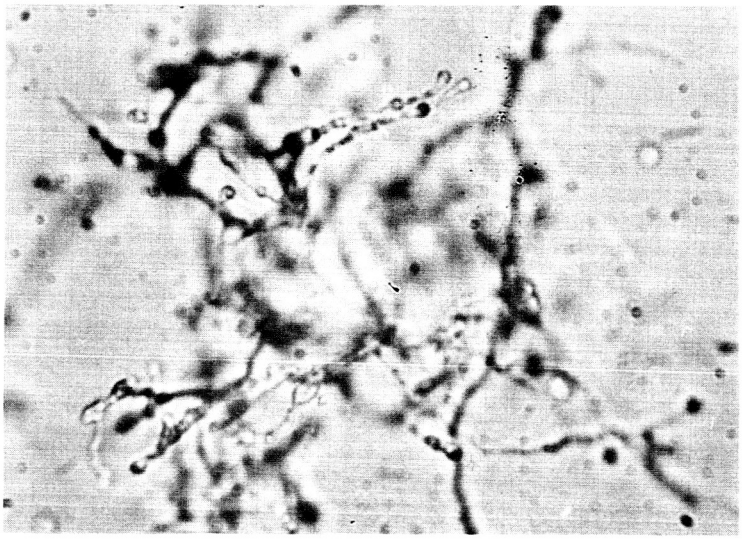
A

Saturated Solution of NaH_2PO_4



B

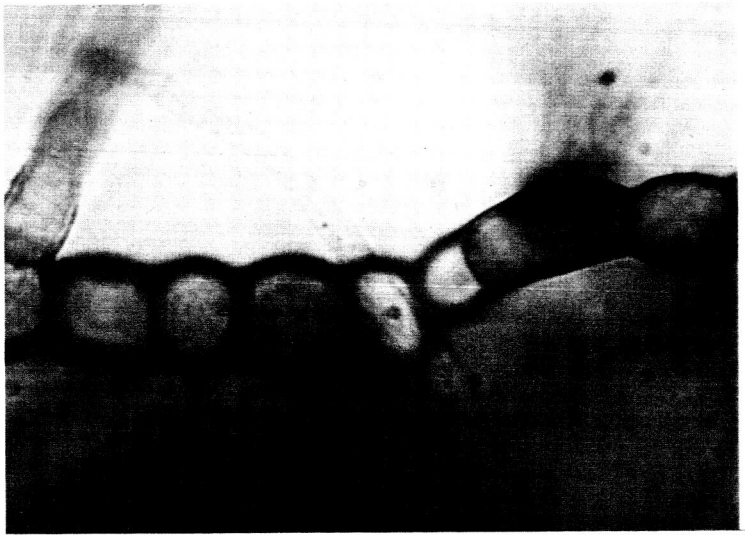
Glycerol with Nutrient



C

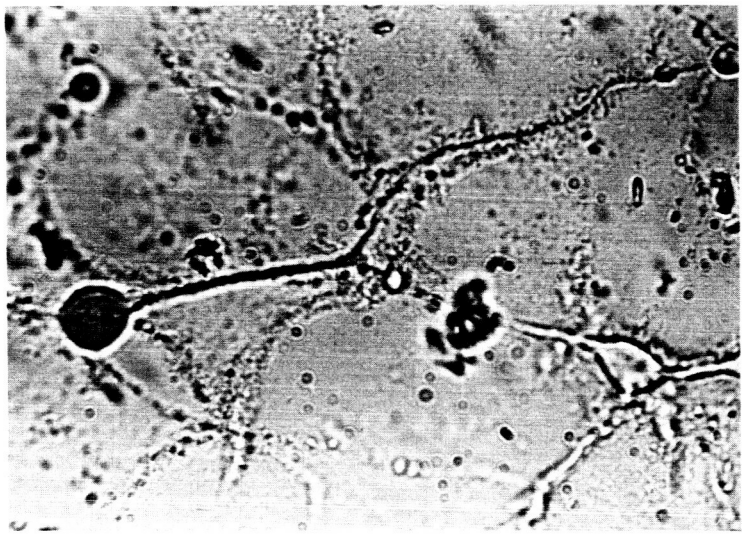
90% Acetic Acid

Plate 8



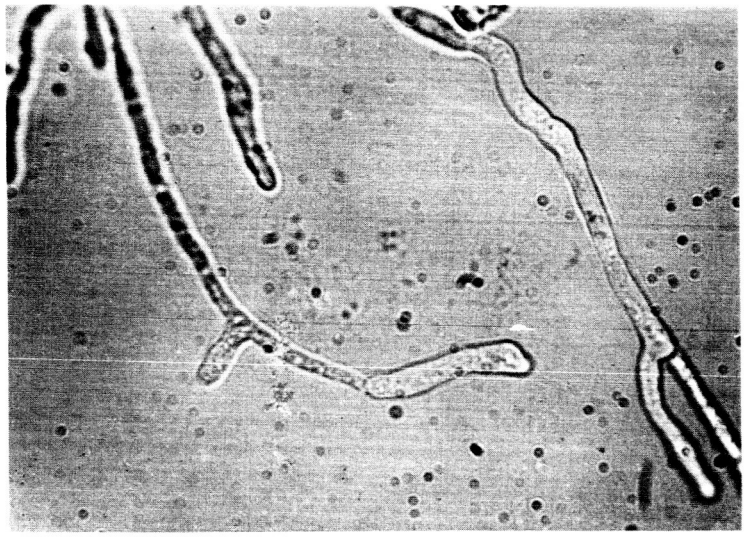
A

KCl



B

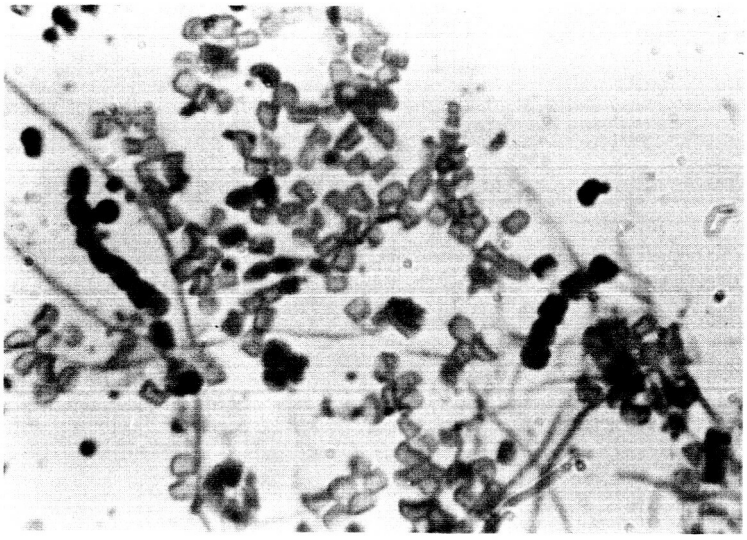
$Ba(OAc)_2$



C

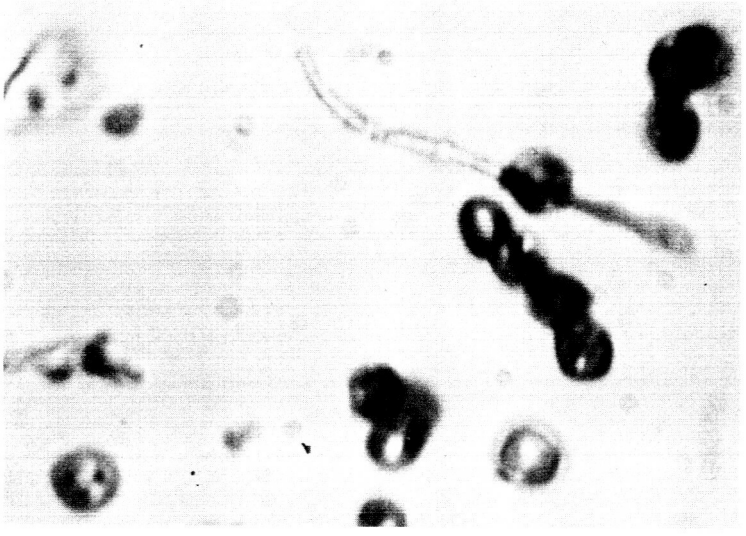
$SrCl_2$

Plate 9



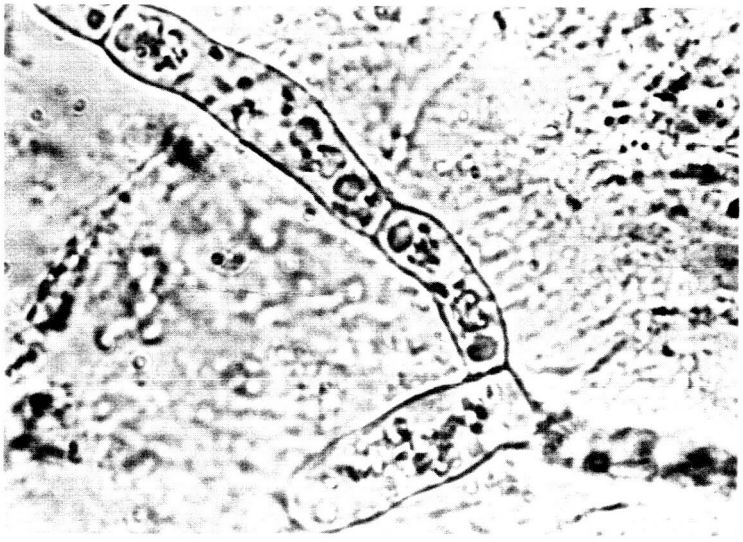
A

Glacial Acetic Acid



B

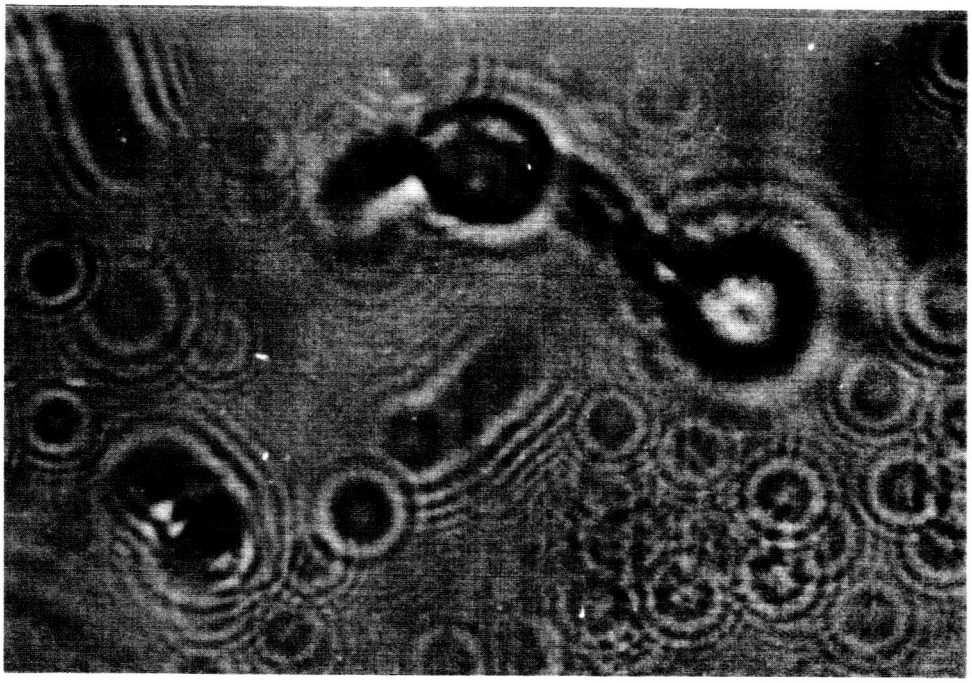
$MgCl_2$



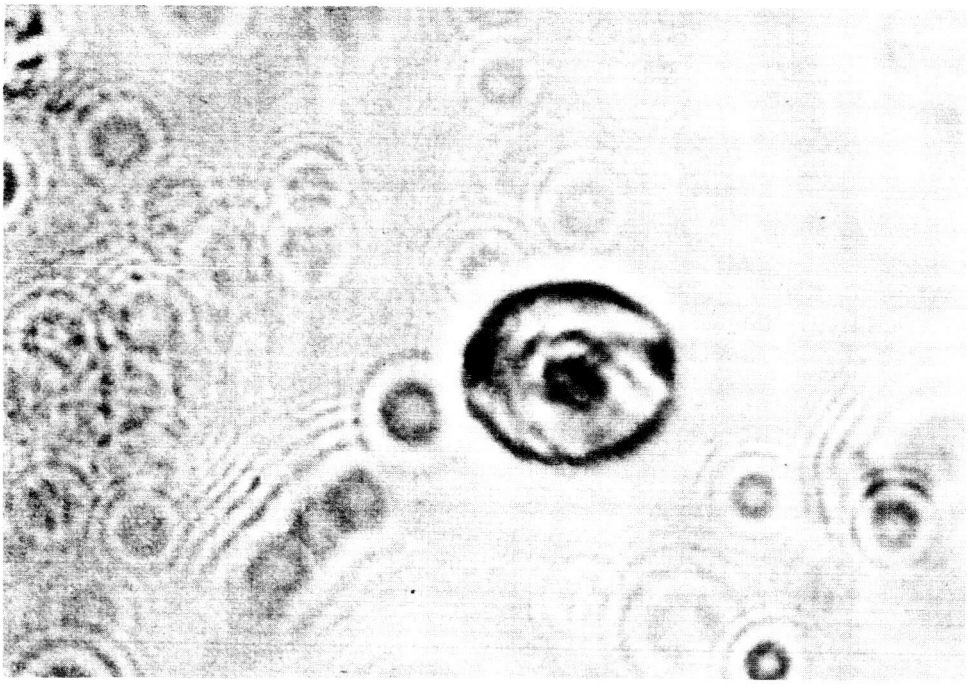
C

$Sr(OAc)_2$

Plate 10



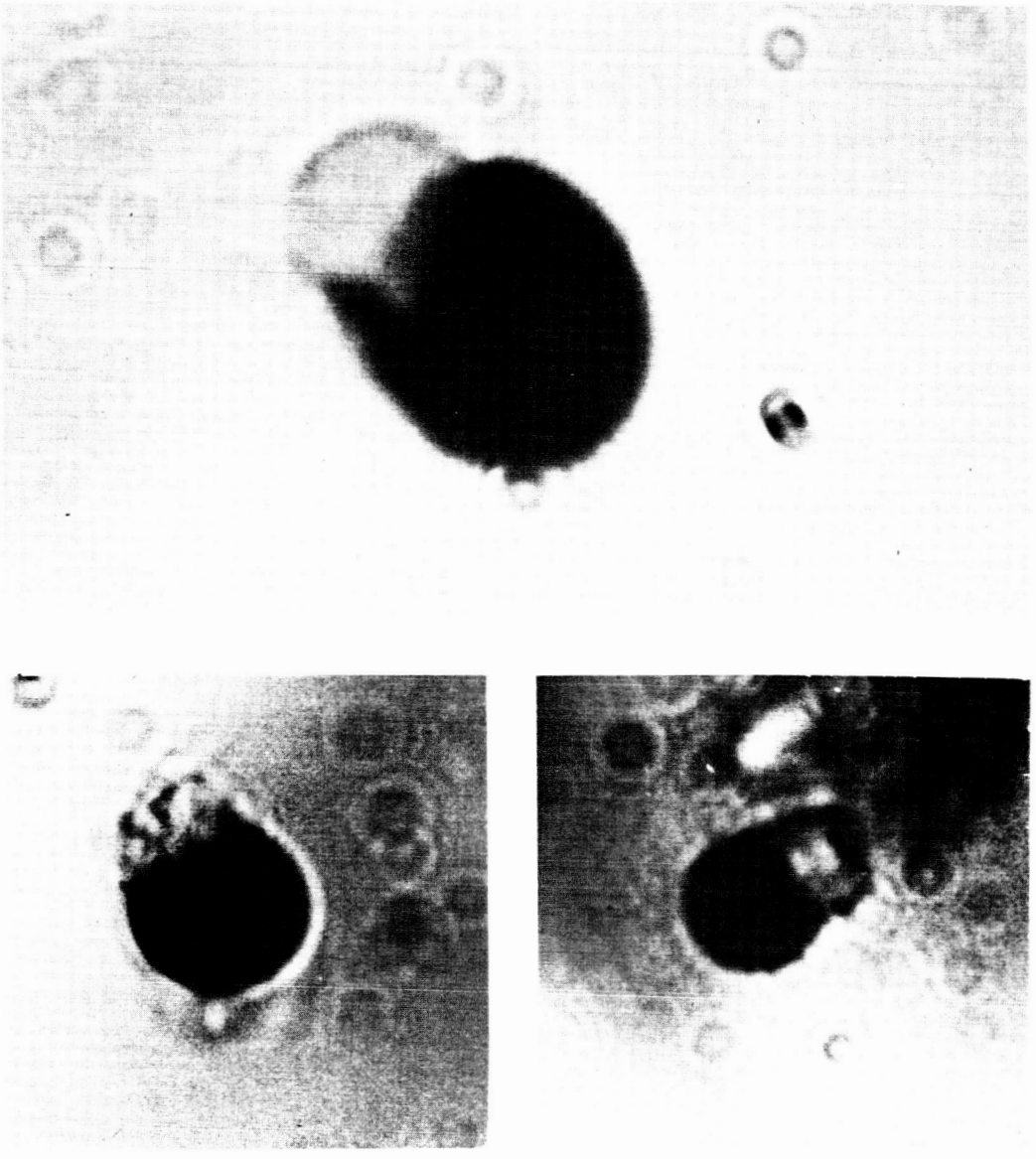
A



B

Germination of Spores in Saturated Solution of NaF

Plate 11



Germination of Spores in a 1 Year Old Culture of 36N H_2SO_4 Kept at a Constant 60°.

Plate 12

V. PERMEABILITY IN RELATION TO ENVIRONMENTAL STRESS: A PRELIMINARY REVIEW OF STATUS

A. General Considerations

The entire history — evolutionary and developmental — of any and all forms of cellular life involves some phase when a cell is immediately surrounded by a non-cellular environment. More generally, many organisms — most, in fact — exist as cells or small cell aggregates in a non-cellular and essentially non-biological fluid. Even those organisms that represent the current products of selection for the dry land environment retain comparatively vulnerable internal cell surfaces, no matter how extensively chitinized, cutinized or cornified they may be. Finally, even in the consideration of homeostatic systems such as Man, organized cell aggregates are surrounded by body fluids which they share in common with many other cells and tissues, as part of the so-called "mileau interieur" (after Loeb).

Any disturbance which is transmitted via the environment into the cell must first encounter the membranes that comprise its surfaces, both external and internal. Unless a given physical or chemical factor is entirely specific to some non-membrane molecular species, process, or system within the cell (e.g. DNA or glycolysis), it must interact with the membrane. If the said factor is reactive, excitatory, disruptive, or capable in any way of causing a pertubation at the macromolecular level, in all probability it will do so at the membrane.

Furthermore, although a chemical or physical stimulus may in some instances pass the membrane without disturbance, the maintenance of the lipoprotein system may be impaired. When a chain of disturbances reaches

the membrane, it is likely to be reinforced (positive feedback), leading to cellular degeneration unless prevented by a definite, adaptive, compensatory, or reparative system.

Finally, within the membrane resides the "point of decision" with reference to tolerance vs resistance. Does the cell selectively exclude excessive concentrations of H^+, OH^-, NH_4^+, heavy metals, or solutes generally as it carries on its normal traffic in material across the membrane? Or does the cell accept foreign substances, and then sequester, precipitate, transform, or immobilize then as noxious agents after (or during) entry? Appropos of these last considerations, do cell membranes (or the protoplast membrane-nuclear membrane continuum) respond to stress in any manner that could be reasonably interpreted as release of information that, when passed along a suitable "code channel", could "call for" the release of membrane-regenerating or detoxifying enzymes?

Specialized human cells known as lymphocytes comprise a somewhat useful model for demonstrating that membrane-monitored stress responses lead to increases in white cell number (Figure 5).

It is hoped that many of the above questions can be approached most expeditiously by a study of responses to extreme environmental stress in organisms generally.

B. Experimental Background and New Activities

In our program under Contract NASw-767, membranes have only once come under consideration, namely, in the lichen Cladonia. Other programs in this laboratory, however, have been directly concerned with the membrane, using the release of pigment from beet-root tissue as a measure of altered membrane permeability.

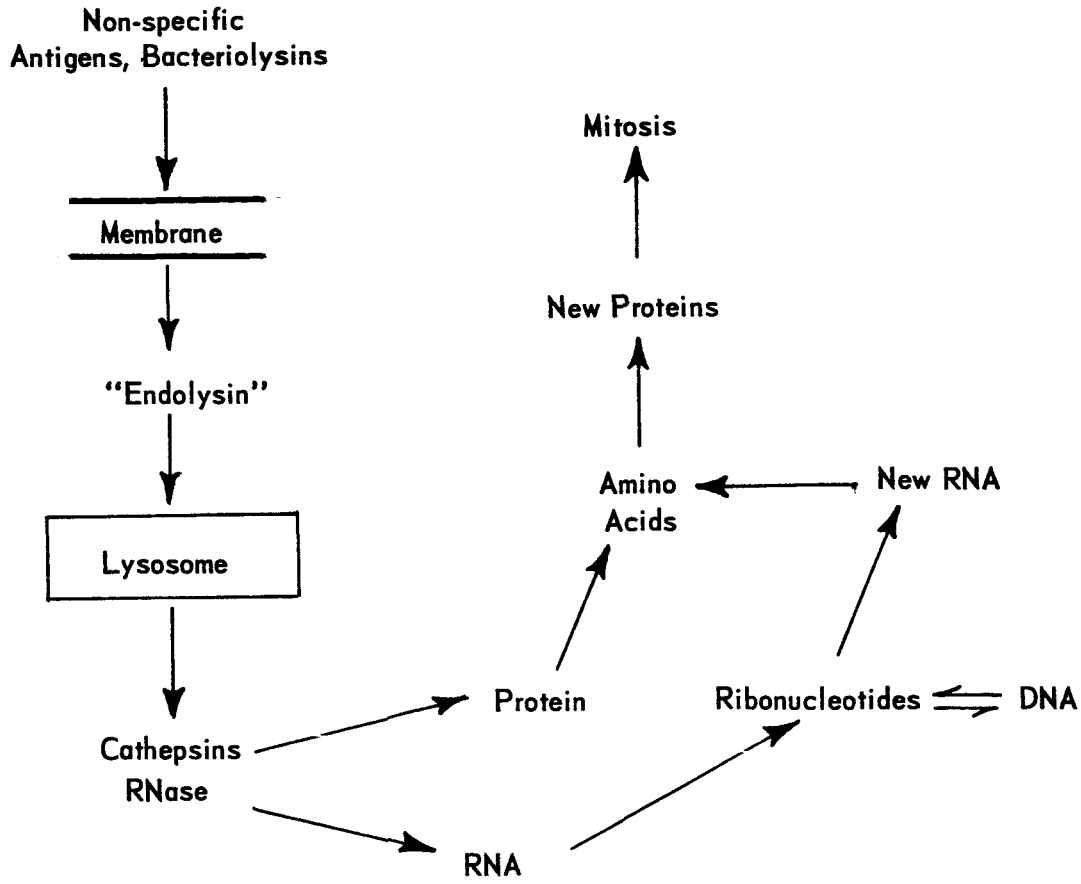


Figure 5 A Model for Membrane Function Under Immunologic Stress in Lymphocytes (After K. and R. Hirschhorn, NYU Medical Center, private communication, 1964-65).

The implication of the membrane in oxygen toxicity was made by Siegel and Gerschman (Siegel, S. M. and R. Gerschman, Physiol. Plantarum 12:314-323, 1959),who observed exudations containing proteins on plants injured by high oxygen pressure. Subsequently, it was shown that organic peroxides at low concentrations are powerful membranolytic agents and that their effects on permeability are closely enough related to growth-inhibition to constitute the inhibitory mechanism (Siegel, S. M., L. A. Rosen, and G. Renwick, Physiol. Plantarum 15:304-314, 1962). Even in the very different situation of growth inhibition by alcohols, the effects of these substances on permeability are sufficient to provide a complete mechanism of inhibition (Siegel, S. M. and L. A. Halpern, Proc. Nat. Acad. Soc. U. S. 51:765-768, 1964). Another aspect of these studies revealed that even thermal lysis of the beet root membrane is a P_{O_2}-controlled process (Siegel, S. M., L. A. Rosen, and C. Giumarro, Nature 198:1288-1290, 1963).

More recent experiments included the demonstration that saline inhibition of winter rye germination can be alleviated by Ca^{++} and O_2 (Siegel, S. M., O. W. Daly and C. Giumarro, Nature 208:1012-1013, 1965). The original account of this study is found in Quart. Rpt. No. 1, 30 Sept. 1963, Section VII (pp. 50-59). Other contract studies generally indicative of or involving permeability factors are:

(a) Growth inhibition in atmospheres containing volatile organic compounds – Quart. Rpt. No. 2, 31 Dec. 1963, Section V (pp. 19-26).

(b) The "second gas" effect in HeLa cells – Semiann. Rpt., 1 Nov. 1965, Section II (pp. 45-46).

(c) Enhancement of O_2 damage of cereals by He and other nobel gas anoxias -

Quart. Rpt. No. 2, 31 Dec. 1963, Section VIII (pp. 34-35),

Quart. Rpt. No. 4, 1 July 1964, Section IIIA (pp. 8-13),

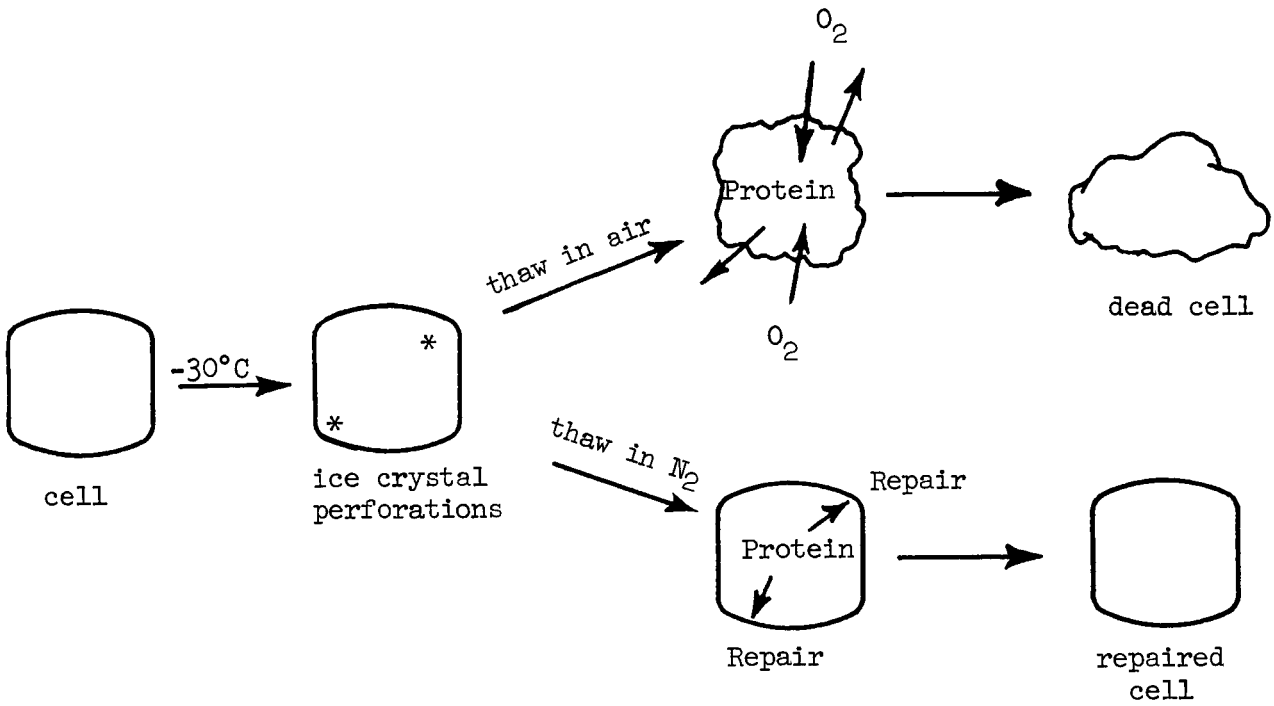
Semiann. Rpt., 1 Nov. 1965, Section IIB (pp. 47-66).

(d) Relation of P_{O_2} to cold injury – Quart. Rpt. No. 4, 1 July 1964, Section IIB (pp. 4-7).

(e) Uses of stain techniques for the study of lichens under stress – Semiann. Rpt., 1 Nov. 1964, Section IV (pp. 20-26).

In essence, (a) and (b) above lead to the conclusion that growth inhibition may be effected in O_2 atmospheres which contain two classes of substances not likely to react chemically with cellular components. First, inhibition by hydrocarbons is more severe the higher their boiling points (e.g. methane not at all, heptane highly toxic), presumably because the higher homologs are fat solvents. Second, among the noble gases, He intensifies the inhibitory effect of moderately hyperbaric O_2, an effect most likely to involve enhanced access of O_2 to the cell interior. Item (c) adds the point that He as an anoxic environment does not show its adverse effect until O_2 is once more introduced at air-level partial pressure.

Item (d) suggests further membrane permeability-P_{O_2} relations by showing that tolerance to diurnal freezing (-30°C, tissues wholly frozen) increased as ambient O_2 level fell to zero, as indicated below:



Finally, item (e) was originally viewed as a possible convenient diagnostic tool until it was found that lichen respiration was readily measurable. It remains a powerful tool for correlating permeability with various injury states. The value of this approach even when stain reactions are gauged subjectively has been established (Figure 6).

We now propose to study more extensively the various stress factors, which have been and are yet to be encountered, using qualitative techniques for assessing changes in the membrane. We shall first study the beet root system within its limits of usefulness, and then, perhaps, quantitate dye uptake in more resistant forms such as lichens.

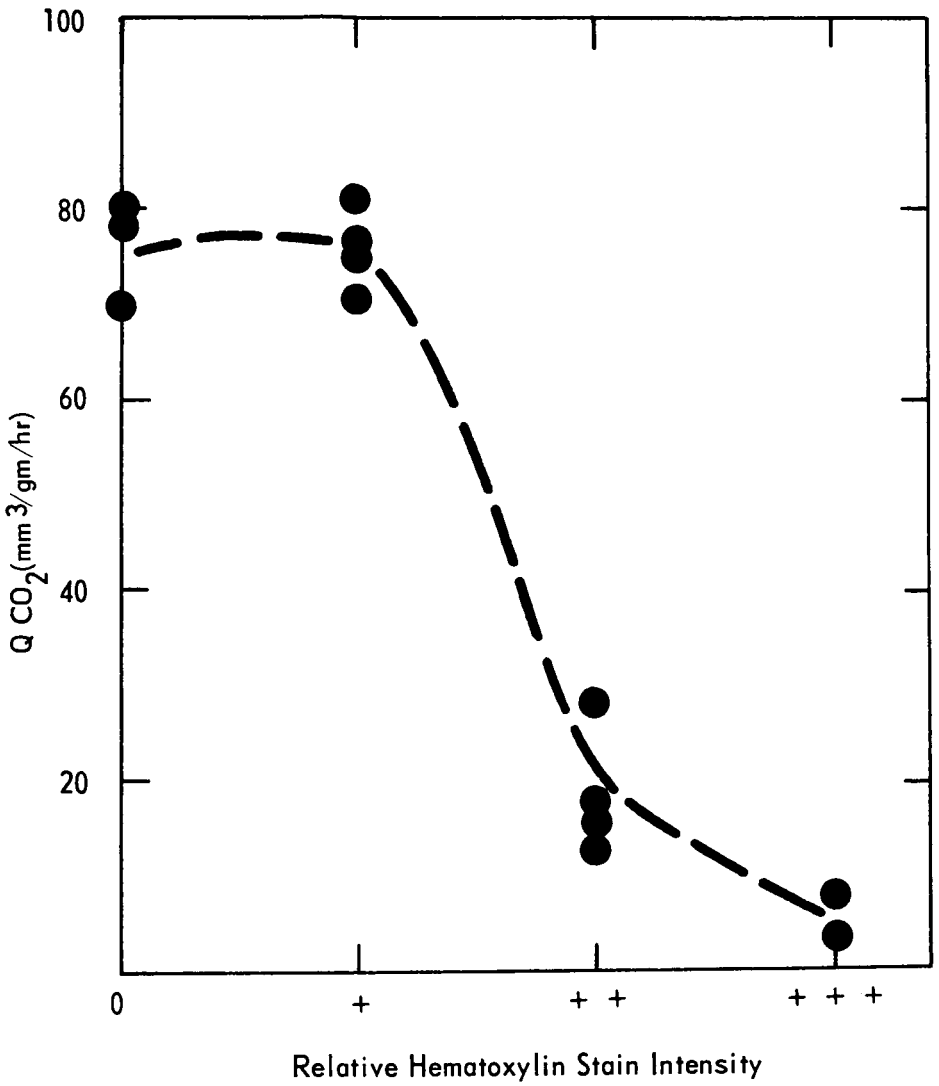
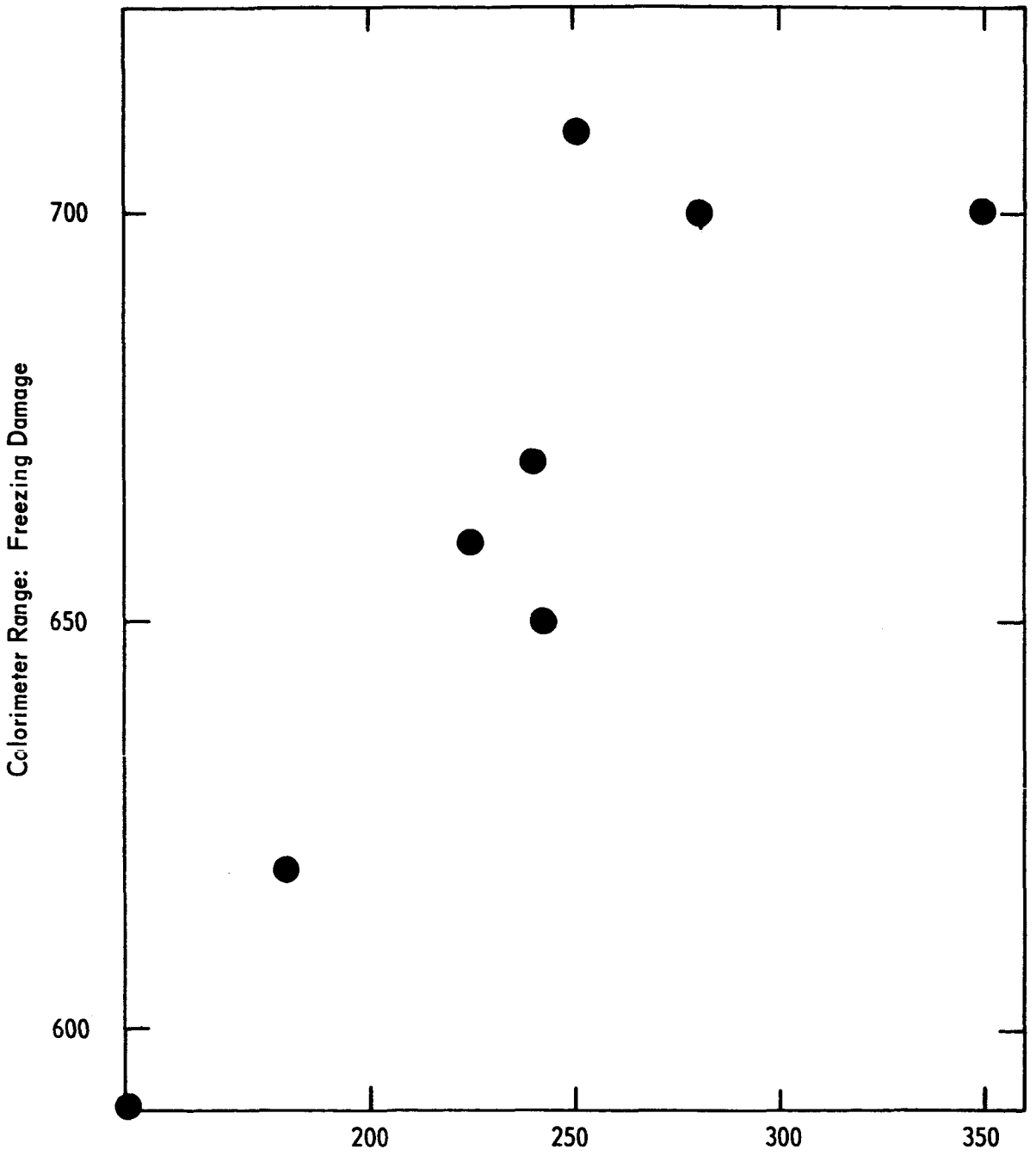


FIGURE 6 RELATION OF RESPIRATORY ACTIVITY TO HEMATOXYLIN STAINING OF CLADONIA SUBJECTED TO VARIOUS STRESSES: FREEZING, BOILING, TOXIC CHEMICALS.

A preliminary experiment was set up using discs cut from 8 different beet roots. The differences in the effects on these samples of a single exposure to -30°C for 4 hours were not great, but were colorimetrically discernible in terms of the extent of pigment leakage. Samples from the same specimens were also immersed in 1 M NH_4OH, and leakage of the pigment followed. When the points for the samples from the several individuals were co-plotted as Freezing Damage vs M NH_4OH Damage, the correlation revealed was highly gratifying (Figure 7).



Colorimeter Range is Expressed in Klett Optical Units Using Filter 54

FIGURE 7 THE CORRELATION BETWEEN FREEZING DAMAGE AND NH_4OH DAMAGE IN BEET ROOT TISSUE.

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